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APPENDIX

A

OPINION

Unravelling the general properties of siRNAs: strength in numbers and lessons from the past

Jonathan Hall

The optimal use of small interfering RNAs (siRNAs) requires an understanding of their general properties, and particularly their selectivity and potency. However, it is often difficult to distinguish the properties of individual siRNAs from their general properties as a class of molecule. The analysis of large sets of siRNAs is one solution. Moreover, years of research into the general properties of antisense oligonucleotides have provided some valuable pointers for designing experiments to unravel the general properties of this new generation of gene-silencing oligonucleotides.

RNA INTERFERENCE (RNAi) represents one of the most powerful biological tools ever to be introduced. It provides a simple, rapid, inexpensive, selective method of gene inhibition with high success rate. Gene-specific RNAi screens in cells or model organisms generate data that link a specific gene to a given biological process. Moreover, genome-wide screens that use a library of individual oligoribonucleotides to knock down each gene return massive amounts of biological information. The results from the first genome-wide libraries of RNAi reagents — dsRNAs in the model organism *Caenorhabditis elegans*[1] and in *Drosophila melanogaster* cell culture[2] — are now available.

Each dsRNA that is specific for a given gene in model-organism RNAi libraries is typically hundreds of nucleotides in length. Each of these dsRNAs is enzymatically processed into a large population of SMALL INTERFERING RNAs (siRNAs), which downregulate levels of the target mRNA. Intuitively, this large number of individual siRNAs should allow each dsRNA in a library to knock down the homologous gene with high sequence specificity and high potency (functionality). However, in most mammalian systems, long dsRNAs seem to induce a toxic INTERFERON RESPONSE. Nonetheless, siRNAs themselves do not elicit this response, presumably because they are too short (see REF. 3).

Within months of the first reports that siRNAs could be used to inhibit gene expression in mammalian cells, a small series of articles and commentaries had proclaimed that this approach was superior to alternative ANTISENSE techniques[4,5]. At miniscule concentrations, siRNAs were said to provide an almost-perfect success rate and an extraordinary specificity, therefore opening the doors to new therapies and to new techniques for genome-wide functional analysis[6].

However, as the application of siRNAs started to become more widespread, reports of shortcomings of the technique increased: these mainly involved associated toxicity, poor levels of inhibition and numerous 'off-target' effects. Many of these reports were contradictory, with conclusions from one study being strongly contested by the next. Here, I argue that these perceived disparities result from the misconception that the behaviour of one oligonucleotide under a certain set of experimental conditions describes the behaviour of this class of molecule under a broad range of conditions. Oligonucleotides do indeed share common properties as a class, but each sequence will also have individual properties, such as potency and stability, that are determined by the specific nucleotide sequence. This has not presented a hurdle for the routine laboratory use of siRNAs as a biological tool, as reagents are usually characterized before initiating extensive experimentation. Reagents that perform unsatisfactorily are simply replaced.

However, genome-wide libraries of human RNAi reagents for high-throughput gene screening will comprise a single (or a small number of) siRNA(s) per gene, applied to cells at relatively high concentrations to ensure potent effects (FIG. 1). So, for each targeted gene, a single siRNA needs to be selected from thousands of candidate siRNAs. Therefore, each siRNA should be potent and specific. The size of such libraries prohibits the characterization of individual siRNA members before experimentation, and, therefore, the design of a genome set needs to take into account the general properties of siRNA oligoribonucleotides as a class. Two such properties are crucial: selectivity at the single-nucleotide level and potency.

The first large-scale gene-knockdown experiments with short hairpin RNAs (shRNAs) in mammalian cells confirm that gene-screening techniques can yield insight into mammalian gene function: in 1 case, 4,873 genes were screened in retroviruses to identify new genes that interfere in the proteasome pathway[7], and in the other, 7,914 genes were screened using a library of shRNA pools to uncover 5 new modulators in the p53 pathway[8].

Here, I argue that to best learn about the general properties of siRNAs that induce RNAi, several experiments need to be performed from which large homogeneous data sets can be generated. From the analysis of such data sets, patterns of general behaviour emerge that are not apparent from the

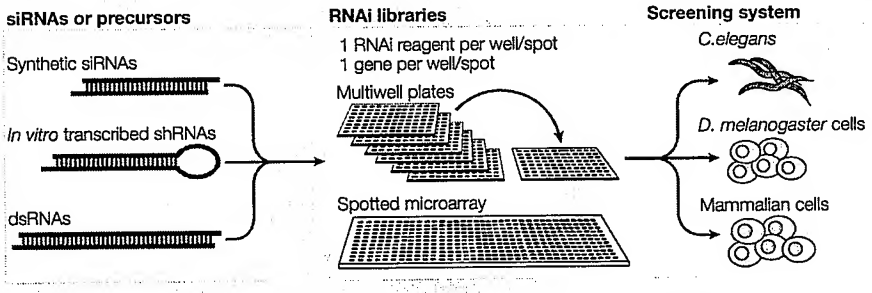


Figure 1 | **Genome-wide screens.** So far, large-scale RNA interference (RNAi) screens have been performed in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian cells. Libraries of RNAi reagents have been created from various sources: small interfering RNAs (siRNAs) are synthesized chemically or prepared enzymatically from primers with T7-RNA polymerase[54]; SHORT HAIRPIN RNAs (shRNAs) are prepared by transcription from Pol II/III promoters and are converted to siRNAs intracellularly by the Dicer enzyme[55,56]; long dsRNAs are prepared by PCR and are processed into siRNAs by Dicer. For each targeted gene, 1–5 siRNAs, or siRNA equivalents, are stored in individual wells of microtitre plates. Reagents are transferred using robotics into microtitre plates that contain living worms or cells, or onto the surface of arrays before the addition of cells.

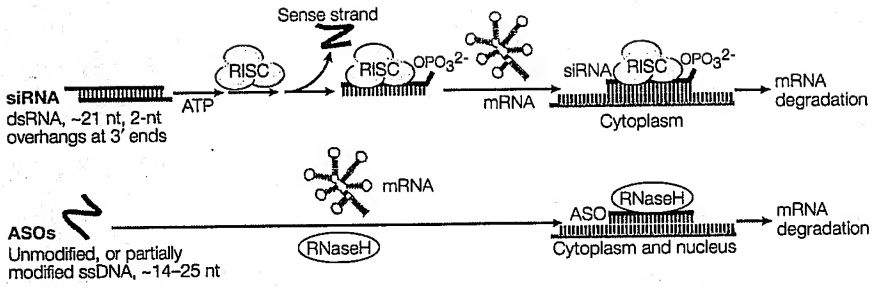


Figure 2 | **Gene inhibition by antisense oligonucleotides and small interfering RNAs.** Small interfering RNA (siRNA)-induced degradation of mRNA in the cytoplasm comprises four principal steps[57]: ATP-independent incorporation of siRNAs into the multi-subunit RNA-INDUCED SILENCING COMPLEX (RISC; possibly in association with the ribosome[58,59]); ATP-dependent unwinding of the siRNA duplex; ATP-independent binding to mRNA; and irreversible cleavage by RNase-III-type activity as the first step of mRNA degradation. Antisense oligonucleotides (ASOs) function as gene inhibitors by one of several mechanisms, depending on the chemical composition of the oligonucleotide. The most commonly used examples are partially modified ASOs, which are delivered to cells in single-stranded form and induce RNase H to bind to the mRNA–oligonucleotide complex in the cytoplasm and nucleus. RNase H cleaves the mRNA progressively. Both processes occur with catalytic amounts of oligonucleotides, although under conditions of cellular transfections, oligonucleotides are often present in excess quantities over the target mRNA. The principal difference between the two mechanisms is the nature and role of the enzymes. However, the interaction of both these types of oligonucleotide with the mRNA follows the rules of Watson–Crick recognition.

behaviour of small numbers of siRNAs in isolated experiments. Moreover, I argue that much can be learned from the antisense field and previous experiments that attempted to address the same questions for antisense oligonucleotides (ASOs; FIG. 2). I review recent work on mismatch selectivity, microRNA (miRNA)-based inhibition, off-target effects and the mechanism of action of the RNA-induced silencing complex (RISC) to illustrate examples of both the individual and the general properties of siRNAs. I also highlight the value of previous antisense research to this area and summarize what we currently know about the general properties of siRNAs (for a recent comprehensive review of advances in RNAi technology, see REF. 9).

Specificity

Single-nucleotide selectivity. Probably the best guide that we have to what determines the nucleotide specificity of siRNAs is the body of work that is already available on the specificity of ASOs (BOX 1). In general, these studies show that oligonucleotides are not 'specific', but rather are 'selective'. So, although a single mismatch in an ASO could theoretically lead to a 500-fold decrease in target affinity[10], in the whole-cell environment, several compensatory factors, such as higher concentrations of highly homologous mRNAs, can result in unintended inhibition of non-target genes (see examples in REFS 11,12). Such off-target effects strongly depend on oligonucleotide length, chemical modification, target accessibility, percentage homology and concentrations of reagent and mRNA[10]. Excellent single-nucleotide selectivity using ASOs is possible, but it requires careful experimental optimization[13].

Similar to an ASO, a siRNA or a shRNA should have a maximum number of mismatches to all other potential binding sites in the transcriptome to ensure the highest possible sequence selectivity. Therefore, the design of a selective inhibitor requires an understanding of the general behaviour of siRNAs towards binding sites with only a few mismatches.

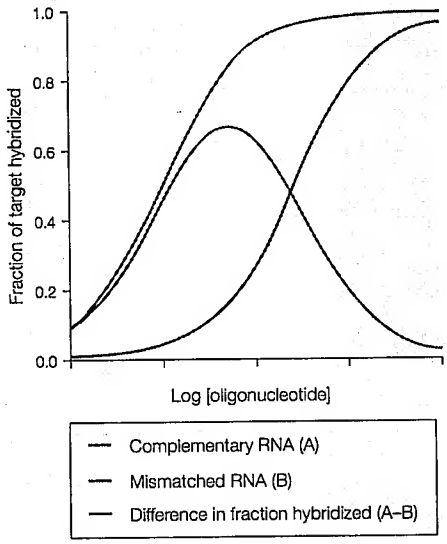
Early publications on siRNAs optimistically asserted that a single-nucleotide mismatch was sufficient to render a siRNA duplex inactive in mammalian cells[14,15]. Recent publications have now demonstrated experimentally that such single-nucleotide selectivity is attainable. For example, a mutant disease-causing allele of the spinocerebellar ataxia type 3 (Machado–Joseph disease; *MJD*) gene was selectively silenced in cell culture[16] by incorporating a mismatch at position 10 of the target site. Selective silencing of alleles of the muscle acetylcholine receptor (*ACHR*) has also been reported[17], with a significant but incomplete degree of selective inhibition of the mutated gene observed at the protein level (83% inhibition compared with 37%) after incorporation of a mismatch at position 9. So, as for ASOs, siRNA selectivity at the single-nucleotide level is possible, but it requires extensive optimization.

A broader investigation of single-nucleotide selectivity using 10–20 siRNAs supported these observations[18]. In this case, siRNAs were designed with single base-pair mismatches to the mRNA target site at 6 different 5′ locations and 2 different 3′ locations. Only the 3′ mismatches showed significantly less inhibition compared with the perfectly matched siRNA. Chiu and Rana[19] also found 2 examples of mismatches at the 3′ end providing superior selectivity. By contrast, a systematic analysis of shRNAs with single base-pair mismatches to the 21 positions in a target site in the mRNA of the human immunodeficiency virus 1 *Gag* gene showed that mismatches at the central target positions 9–11 and at positions 4 and 16–18 were the most discriminatory[20].

Collectively, these efforts have not fully explained the general behaviour of siRNAs with respect to single-nucleotide specificity. They demonstrate convincingly that fully complementary reagents are the most inhibitory and that mismatches at terminal locations provide only minor discrimination. However, the data sets are too small to conclude whether, in general, there is a minimum number of mismatches required to ensure specificity for a target gene under a broad range of conditions or if different regions of the oligoribonucleoides (5′, 3′ regions, target cleavage site) contribute differently to selectivity. Nevertheless, bioinformatic analyses that are aimed at identifying the mRNA targets of miRNAs do indicate that some regions in such oligoribonucleotides might be more important than others[21], and new experimental evidence supports this[22]. Regardless, the few studies discussed above indicate that if RNAi libraries are designed for high-throughput screening, reagents probably need to have multiple mismatches to all other potential binding sites in the transcriptome to minimize off-target effects.

siRNAs and miRNAs. An added complication to siRNA design stems from recent suggestions[23] that, similar to miRNAs, siRNAs might be able to bind to partially homologous sites that contain insertions and deletions and still inhibit gene expression. miRNAs are a second class of small regulatory RNAs that inhibit gene expression by non-degradative translational attenuation. Scacheri *et al.*[23] proposed that siRNAs might act through a miRNA-like mechanism to explain their finding that a subset of the siRNAs designed to knockdown the *MEN1* (multiple endocrine neoplasia I) gene significantly upregulated *p21* (cyclin-dependent kinase inhibitor 1A; *CDKN1A*) and *p53* (tumour protein 53; *TP53*) genes. However, bioinformatic analyses did not reveal any probable miRNA targets, so the real origin(s) of these effects remain unknown.

Box 1 | Selectivity of oligonucleotides



The figure shows the theoretical *in vitro* hybridization profiles of an oligonucleotide that is present in excess to a fully complementary RNA (A) and to a partially complementary RNA (B) that bears a mismatch. In each case, the fraction of bound RNA varies with the affinity of the oligonucleotide for the target, and the concentration. The selectivity of the oligonucleotide for RNA (A) over (B) is therefore determined by concentration and affinity and, for any two given RNAs, is possible within a concentration range that is defined by the line (C). As the horizontal distance between the curves (A) and (B) increases, the concentration range for which good selectivity can be obtained is widened. So, to maximize the selectivity of an oligonucleotide for its complementary RNA in the presence of highly homologous RNAs, an antisense sequence should be designed to have the greatest difference in affinity (that is, the highest melting temperature difference; ΔTm) with the next closest predicted binding homologue. So, the oligonucleotide should have the maximum affinity for the target and a maximum number of mismatches to any other closely related homologues, such that curve (A) can be shifted to the left and curve (B) to the right.

In a gene-knockdown experiment that uses oligonucleotides that induce mRNA cleavage, specific target cleavage rather than specific target binding, is the goal. Factors that affect target cleavage are numerous and complicated. These factors include the concentrations, and secondary and tertiary structures of target and non-target mRNAs, as well as sequence motifs. Consequently, theoretical selectivity that is based on the fraction of the target that is bound might not reflect selectivity of the fraction of the target that is actually cleaved.

Nevertheless, for antisense oligonucleotides (ASOs) hybridization thermodynamics is a crucial factor for efficient, selective gene inhibition and explains cases in which good selectivity has been observed[49]. In recent years, several structural modifications to ASOs that provided improved mismatch specificity were introduced[50], and, in contrast to small interfering RNAs (siRNAs), the length of ASOs can be varied to further improve selectivity[51].

The situation for siRNAs is different. The RNA interference (RNAi) mechanism involves multiple discrete enzymatic steps before hybridization of siRNA with target mRNA. Moreover, RNA-induced silencing complex (RISC) elements that are responsible for mRNA cleavage might even aid the hybridization process. By contrast, ASOs probably recruit RNase H only after hybridization. Furthermore, emerging evidence from microRNAs (miRNAs)[22] indicates that different regions of a RISC-loaded guide strand have different roles in RNAi. Importantly, the 5′ half of the guide is more involved in target recognition, and therefore is the best location in which to incorporate mismatches to other highly homologous mRNA binding sites. Although these factors taken together imply that the above model might be less applicable to siRNAs than ASOs, Pancoska *et al.*[52] have provided experimental evidence that hybridization thermodynamics between the siRNA guide strand and the mRNA is an important factor for efficacious gene inhibition and that the excellent selectivity that was observed in the experiments of Semizarov *et al.* included a sequence design that incorporated hybridization thermodynamics. In conclusion, the mechanism of action of the individual oligonucleotides should define the best approach for investigators to use for target-specific inhibition. Figure modified with permission from REF. 53 © (1993) CRC Press.

A handful of papers have described experiments that were performed to specifically investigate gene inhibition by small RNAs that act at partially complementary mRNA sites. In one study, co-transfection of a plasmid that codes for *miR-30*, a naturally-occuring miRNA that is expressed in mice and humans, and a luciferase reporter gene that carries 4 identical, consecutive partially homologous target sites in the 3′-untranslated region (UTR), resulted in expression of *miR-30* and inhibition of luciferase activity without mRNA degradation[24]. If the bulged target sites were exchanged for a single fully complementary site, luciferase activity was inhibited through mRNA degradation. These experiments showed that miRNAs can function as siRNAs. But can siRNAs function as miRNAs? A second publication from the same group[25] answered this question. Transfection of a siRNA homologous to the *Drosophila melanogaster Nxt* gene inhibited translation of luciferase that has eight identical repeated miRNA-like target sites, without affecting mRNA levels.

Independent confirmation that siRNAs can downregulate genes by interacting with bulged target mRNAs came from another group[26]. Four consecutive target sites that were predicted to form bulged duplexes with a *CXCR4* (chemokine (C-X-C motif) receptor 4) siRNA were introduced into the 3′ UTR of a luciferase gene. Again, good inhibition of luciferase activity was achieved without any observed lowering of mRNA levels. Additional experiments to define the rules of the interactions led the authors to suggest that the sequence of the bulge is not a principal determinant, that a minimum amount of free energy for binding the first 8 nucleotides of the miRNA 5′ region is necessary for translational inhibition and that complementarity in the 3′ end of the miRNA is less crucial than at the 5′ end[22].

Together, these three studies show that siRNAs can downregulate genes in a miRNA-like fashion. However, multiple repeat copies of the target site were required to see efficient translational inhibition. This in itself should ensure that such interactions are unlikely to pose particular problems with respect to siRNA design. However, Saxena *et al.*[27] reported that siRNAs with partial complementarity to a single site in the coding regions of *p21* and *geminin* (*GMNN*) caused translational inhibition. In this case, similar levels of inhibition were observed at complementary and bulged mRNA target sites, and inhibition occurred through interaction at a single binding site, located in the coding region. If more examples that are similar to this emerge, then this non-complementary interaction will indeed pose problems for the design of selective siRNA reagents until the rules that govern the interaction are better understood.

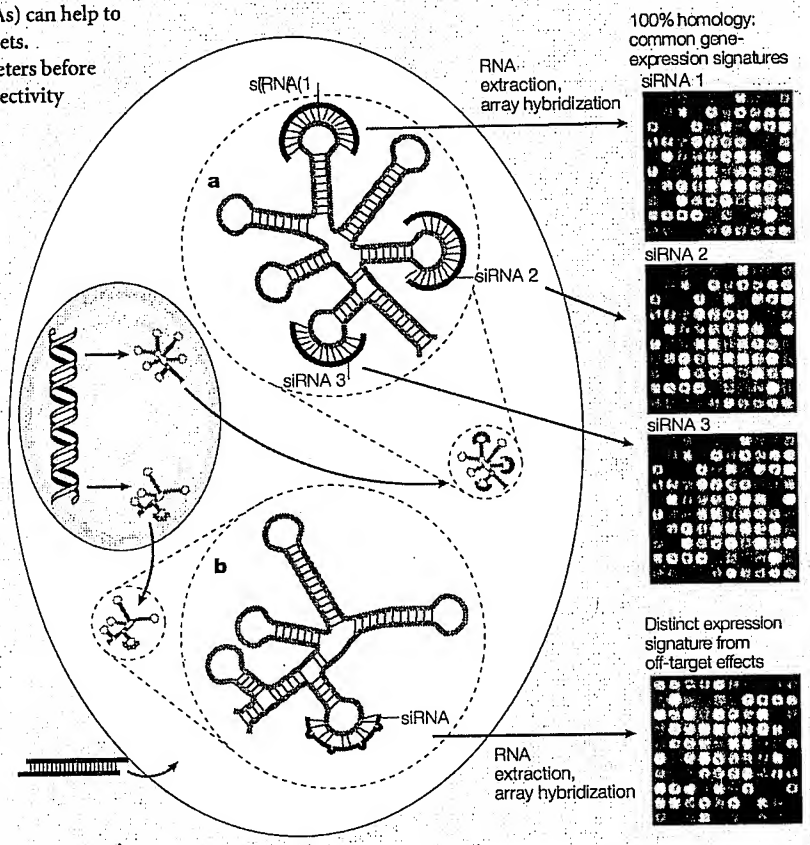
Genome-wide studies. DNA arrays provide a snapshot of the mRNA levels of all cellular genes represented by oligonucleotide probes that are present on the array. Therefore, they provide a comprehensive means to query the selectivity of an individual oligoribonucleotide in the cell, albeit with the limitation that the technique only records changes in mRNA levels: that is, translational inhibition is not directly observed. Such studies can also

Box 2 | Investigating the selectivity of small interfering RNAs using gene-expression profiling

Careful sequence design of small interfering RNAs (siRNAs) can help to ensure that reagents are selective for intended mRNA targets.

For example, Semizarov *et al.*[30] optimized several parameters before carrying out array hybridizations to achieve maximum selectivity of siRNAs. Three target genes were selected. Multiple characterized siRNAs per mRNA were used: various 19-nucleotide sequences against the target genes were ordered by decreasing homology against the next best hit within the transcriptome, and only those with fewer than 15 nucleotides to the next best hit were selected. Those siRNAs with the highest predicted difference in calculated affinity between the match target and the next most probable predicted mismatched mRNA target were then chosen. The authors applied the same selection criteria to the mismatched negative controls. Transfection of siRNAs at 100 nM usually led to the induction of selected apoptosis and stress-response genes. However, a short-dose response and time-course study showed that transfection of the siRNAs at 20 nM was equally inhibitory against the intended target, whereas most of the previously observable nonspecific effects disappeared, and, therefore, siRNAs were used at this concentration. Transfection of multiple siRNAs that targeted any one of the given genes, as depicted in (a), then resulted in highly correlated gene-expression signatures.

Under non-optimized experimental conditions, siRNAs, like antisense oligonucleotides, bind and inhibit the expression of genes that contain partially complementary target-binding sites (b). A gene-expression profile that uses DNA microarrays yields a distinct expression signature that is associated with the 'off-target' effects of the siRNA (b)[29].



provide gene-expression signatures for groups of siRNAs that are directed to distinct sites of a given gene. In these cases, modified expression of a common set of genes from the relevant pathways is observed in addition to target-gene inhibition.

Four publications describe microarray experiments performed to address selectivity of siRNAs. In the first, microarray analyses of transfections of *GFP* siRNAs into a *GFP*-expressing cell line showed that out of 36,000 genes, only *GFP* was consistently knocked down, which indicated that siRNAs were highly specific[28]. The contradictory results and conclusions of two more papers in 2003 added fierce controversy to the debate on siRNA specificity. Genes with well-characterized biology were targeted in both studies (BOX 2). In one, 24 siRNAs, designed by standard selection rules[14] and with fewer than 18 nucleotides of homology against any other theoretical target sites, were used to silence two genes, *IGF1R* (insulin-like growth factor 1 receptor) and *p38* (REF. 29). Each of the siRNAs produced distinct gene-expression signatures and none of the down-regulated genes was a recognized member of the targeted pathways. The authors suggested that cross-hybridization with partially homologous sites (with as few as 11 shared nucleotides) led to these off-target effects, and that a siRNA sequence that is 'absolutely' specific would therefore be difficult to select.

The microarray experiments of Semizarov *et al.*[30] began after a careful analysis of reagents and experimental conditions. Five siRNAs were used for each of the three target genes. *RB1* siRNAs modified the expression of 2,475 genes in these experiments. All five *RB1* siRNAs had similar gene-expression signatures, which included a modified expression of the target cell cycle and DNA biosynthesis genes. Similarly high correlations were obtained for *AKT1* and *PLK1* siRNAs. siRNAs against different targets showed little overlap in expression signature, confirming that effects were due to specific target downregulation. So, in contrast to the conclusions from the *IGF1R* and *p38* study, these data indicate that siRNAs can be highly specific in mammalian cells with careful siRNA selection, meticulous experimental design and the inclusion of negative controls.

The last publication of the four describes microarray experiments in which luciferase-expressing cells were over-dosed with an anti-luciferase siRNA[31]. Large numbers of non-target genes were up- and downregulated, and 12 of these were selected for more detailed study. A second unrelated siRNA regulated the 12 genes in a similar fashion, and a subset of these was similarly affected by treatment of cells with interferon or with long dsRNAs. As the 12 genes responded identically to 2 unrelated siRNAs, it might be tempting to assume that they are regulated by siRNAs in general. However, these effects might simply have been caused by the chance presence of a single motif in both siRNAs, just as a CpG motif in ASOs or a terminal triphosphate on a siRNA can cause distinct nonspecific effects[11,12,32]. More experiments using more siRNAs together with a set of oligonucleotide controls, such as plasmid DNA or ASOs, are needed to test this hypothesis.

Genome-wide microarray experiments are too sophisticated and expensive to consider as a routine method to assess the selectivity of any given siRNA and to allow researchers to

be sure that phenotypic effects are due to the inhibition of the intended target. There is sufficient choice of other types of control available to do this: multiple siRNAs that are targeted to distinct non-overlapping sites of the same target, cDNA-rescue constructs that are unaffected by the siRNA in question and unrelated siRNA-negative controls. However, microarray studies might be required for siRNAs designed as therapeutic agents to help to assess potential toxicity[33].

Potency

The functionality of si/shRNAs has been one of the most contentious aspects of the field. Oligonucleotide potency, similar to selectivity, is a subjective quality that depends on numerous experimental parameters. So, references to potency make most sense if used in a relative context in which oligoribonucleotides are normalized to a reference, or to each other. Early controversy in the field centred on claims of an almost-perfect success rate with siRNAs. Harborth *et al.*[14] showed that the first siRNAs selected downregulated 14 out of 16 endogenously-expressed genes, including Lamin A/C (*Lmna*) (see also REF 34). Not everyone experienced this success. For example, only 1 out of 5 *Lmna* siRNAs were effective in a subsequent study[35]. Similarly, Holen *et al.*[36], in the first systematic evaluation of the efficacy of multiple siRNAs against the same target mRNA, found that siRNAs showed varying levels of functionality. Another early larger-scale study compared the potency of siRNAs to ASOs in 2 genes (*CD54* (*ICAM1*) and *PTEN*)[37]. Only one-third of the ~80 siRNAs tested were classified as 'active': half as many as the ASOs.

Today, with hundreds of publications that describe the use of siRNAs, it is apparent that many are indeed non-functional, whatever the experimental conditions. This constitutes a serious hurdle for the construction of genome-wide collections as investigators are obliged to include multiple si/shRNAs in a library to ensure efficient knockdown of each targeted gene, adding significantly to the expense of reagents, screening and data analysis[7,8].

Two back-to-back papers that describe how the RISC selects the antisense guide strand have contributed significantly to the understanding of features that confer potency to a siRNA. The affinity of oligonucleotide–mRNA-duplex formation has long been known to be essential for an efficient antisense-based inhibition, and early RNAi work indicated that duplex unwinding is a crucial processing step of both dsRNA and pre-miRNAs. Khvorova *et al.*[38] examined the theoretical thermodynamics profiles of 272 miRNAs and their predicted duplexes with mRNAs. They discovered that the 5′ terminus of the guide strand of the miRNA consistently showed a weaker predicted binding affinity than the corresponding 3′ end. The related enzymatic processing of miRNAs and siRNAs led them to predict that the selection criteria for the siRNA guide strand might be similar for the stabilization of miRNAs by RISC. Visual analysis of thermodynamic properties of 37 active siRNAs showed that the 5′ end of the antisense strand of the potent siRNAs also consistently showed a weaker predicted duplex-binding potential than that of the sense strand. This observation was confirmed with 3 sets of experiments that involved more than 200 siRNAs: the strongest inhibitors all carried the predicted profile. So, the analysis of large data sets, first from miRNAs and then from siRNAs, revealed a truly general property of potent siRNAs. In future, this paper will probably significantly influence the design of siRNA reagents.

Another study that was published at the same time provided supporting evidence for the results reported by Khvorova *et al.* and an explanation as to why this local duplex affinity is a key determinant of RISC selection[39]. Terminal nucleotides were mutated on both strands of a siRNA to change the binding affinity of the oligoribonucleotides: the siRNA strand with the less stable 5′ end is preferentially incorporated into the RISC complex.

A more recent study of 180 randomly selected siRNAs from regions poor in G+C content, targeting 2 genes, has built on the conclusions of these 2 papers[40]. Once again, the large data sets revealed generic traits, including positive and negative determinants. For example, a 2-nucleotide shift in the targeted region can cause a large change in functionality, and approximately 78% of the sequences induced more than 50% silencing. In the sense strand of active siRNAs, an A+U-rich region is observed in the 3′ part: A is often found at positions 3 and 19, and U at position 10. These features were combined and incorporated into an algorithm-based design tool that was aimed at improving the selection of potent reagents.

Three other reports have used large sets of reagents to investigate the general properties of potent siRNAs. A study of 62 siRNAs highlighted several common features of the antisense strand of active siRNA sequences: A or U at the 5′ terminus, G or C at the 3′ end and an absence of G+C stretches of more than 9 nucleotides[41]. Similarly, a study of approximately 150 siRNAs targeted at 22 genes of the PI3K (phosphatidylinositol 3-kinase) pathway revealed a preference for U at the 5′ terminus (position 1) and G or C at position 9 of the antisense strand[42]. In the last of these recent studies, Amarzguioui and Prydz[43] evaluated 46 siRNAs and found that A at position 6 can be helpful, in addition to the A+U-rich region at the guide strand 5′ terminus.

This small group of publications demonstrates how larger-than-average data sets have been used to identify general features of potent siRNAs, and how they have been included into effective selection algorithms. Investigators agree on the importance of an A+U-rich region at the 5′ end of the guide strand, including the terminal position, but, at present, there seems to be little consensus for other positions. It is probable that more consensus sequence motifs will emerge, possibly more complex than at the single-nucleotide level, as data sets continue to grow in size and more sophisticated analysis methods are applied to the data. Ultimately, the use of neural network programmes will be used with such data sets, as was recently described for ASOs[44], and the outcome will be a tool that predicts potent siRNAs with high accuracy.

Glossary

ANTISENSE
DNA or RNA that is manipulated in a laboratory to be complementary to a target mRNA. Antisense techniques are used to inhibit the expression of genes in a sequence-specific fashion.

INTERFERON RESPONSE
A primitive antiviral mechanism that triggers sequence-nonspecific degradation of mRNA and downregulation of cellular protein synthesis.

microRNA
(miRNA). Small regulatory, antisense RNAs (21–25 nucleotides long) that repress the translation of homologous target RNA.

RNA-INDUCED SILENCING COMPLEX
(RISC). A multi-component, ribonucleoprotein complex that cleaves specific mRNAs that are targeted for degradation by homologous dsRNAs during the process of RNA interference.

RNA INTERFERENCE
(RNAi). A process by which dsRNA specifically silences the expression of homologous genes.

SHORT HAIRPIN RNAs
(shRNAs). Small RNAs that form hairpins that can induce sequence-specific silencing in mammalian cells through RNA interference, both when produced exogenously and transfected into the cell, and when expressed endogenously.

SMALL INTERFERING RNAs
(siRNAs). Small antisense RNAs (20–25 nucleotides long) that are generated from specific dsRNAs that trigger RNA interference. They serve as guides for the cleavage of homologous mRNA in the RNA-induced silencing complex (RISC).

Investigators cannot agree on the importance of mRNA secondary and tertiary structure to siRNA potency[40,45]. The importance of secondary structure was elegantly addressed previously for ASOs by cloning an invariant target site into mRNA constructs of well-defined secondary structures, and monitoring the activity of a constant ASO as a function of changing structure: stable RNA structure led to attenuated inhibition[46]. In the same system, active siRNAs behaved similarly, and, therefore, it is probable that target structure also affects siRNAs[37]. A much larger study that used approximately 50 different target constructs confirmed this finding[47]. These results imply that, although including particular sequence motifs when designing siRNAs can optimize their potency, the activity of siRNAs is probably never completely independent from the effects of mRNA local structure.

Conclusions

Investigators have struggled to explain two general properties of siRNAs: potency and selectivity. The more we know about these properties, the better will be the performance of what is already a highly effective and widely used tool. Moreover, a deep understanding of these properties is essential for the design of si/shRNAs for genome-wide screening (FIG. 1). There are now hundreds of publications that describe the use of siRNAs in biology, and although several of these purport to portray the general properties of siRNAs, those that use large data sets can reliably reveal the general properties of these reagents as a class, as opposed to the properties of individual sequences. Furthermore, in many cases, experimental design has been most effective if we have taken heed of lessons from the past[48].

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The author declares that he has no competing financial interests.

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APPENDIX

B

nature biotechnology

REVIEW

Approaches for the sequence-specific knockdown of mRNA

Lisa J Scherer & John J Rossi

Over the past 25 years there have been thousands of published reports describing applications of antisense nucleic acid derivatives for targeted inhibition of gene function. The major classes of antisense agents currently used by investigators for sequence-specific mRNA knockdowns are antisense oligonucleotides (ODNs), ribozymes, DNAzymes and RNA interference (RNAi). Whatever the method, the problems for effective application are remarkably similar: efficient delivery, enhanced stability, minimization of off-target effects and identification of sensitive sites in the target RNAs. These challenges have been in existence from the first attempts to use antisense research tools, and need to be met before any antisense molecule can become widely accepted as a therapeutic agent.

Over the past two decades, the use of nucleic acid–based inhibitors of gene expression (antisense agents) has come in and out of fashion. Initial excitement in this area came in the mid-1980s as synthetic DNA chemistry hit full stride, enabling sequence-specific antisense ODNs to be rapidly synthesized and tested for target-specific knockdown of gene expression. It was quickly realized that certain backbone modifications were necessary for full activity of these compounds and that efficient delivery to target cells was a critical requirement. Chemists quickly addressed these challenges by developing a variety of backbone modifications that stabilized antisense ODNs without inhibiting their biological activities. Delivery challenges were also addressed with the introduction of anionic and cationic lipid formulations for packaging and delivering the net negatively charged ODN compounds to a variety of cells in culture. In subsequent years, however, interest declined because the predicted utility of these compounds as therapeutic agents was slow to materialize and, in fact, remains limited to a handful of compounds.

The second wave of interest in nucleic acid–based inhibitors of gene expression followed the discoveries of catalytic RNAs (ribozymes) in the early 1980s. The full potential of ribozymes for target-specific inhibition of gene expression was not completely realized until the late 1980s and early 1990s when simplified catalytic motifs were defined, making these molecules amenable to chemical synthesis. The exploitation of ribozymes as therapeutic agents also depended heavily upon stabilizing backbone modifications that did not inhibit activity and efficient delivery. Fortunately, these issues could be addressed by drawing on the extensive experience of the antisense ODN field. Ribozymes have an advantage over ODNs in that ribozyme genes can be delivered to cells with plasmid or viral vectors, and ribozyme expression can be controlled with promoter-based expression.

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The most recent explosion of interest in the antisense world followed the discoveries of Mello and colleagues[1] in *Caenorhabditis elegans* in 1998, and of others in mammalian cells in 2001 (refs. 2,3), that double-stranded RNAs (dsRNAs) elicit potent targeted degradation of complementary RNA sequences, termed RNA interference (RNAi). Moreover, it was shown that the active component of the RNAi pathway, termed small interfering RNAs (siRNAs), can be chemically synthesized or expressed from vector backbones, similar to ribozymes. The interest in RNAi has been fueled—to an even greater extent than interest in antisense ODNs and ribozymes—by the completion of the human genome sequence initiative because siRNAs can elicit potent, target-specific knockdown of any mRNA, creating a useful and proven surrogate genetic tool. Although RNAi provides a powerful new tool for targeted inhibition of gene expression, there are, nevertheless, concerns and limitations in the use of this technology as well, including efficient delivery and potential side effects.

There have been important developments in the other areas of antisense technologies, giving the investigator several options, depending upon the experimental system and desired outcome. This article explores the basic mechanisms of action of only the popular antisense inhibitory agents. We then compare the advantages and disadvantages for each class of inhibitory agent. We do not intend to present a comprehensive review of the antisense world, but rather to provide a framework for thinking about which agent best matches the goals of an experimental or therapeutic application.

Antisense oligonucleotides

The notion that small ODNs could be used to specifically inhibit gene expression was first put forth in 1978 by Zamecnik and Stephenson[4,5]. Their studies demonstrated that a tridecamer (13-mer) ODN complementary to terminally repeated sequences in Rous sarcoma virus (RSV) long terminal repeat (LTR) inhibited both RSV translation in a cell-free system and viral replication in cultured cells[4,5]. It took several years after these elegant experiments for investigators to begin to fully realize the potential of antisense-mediated gene inhibition. With the

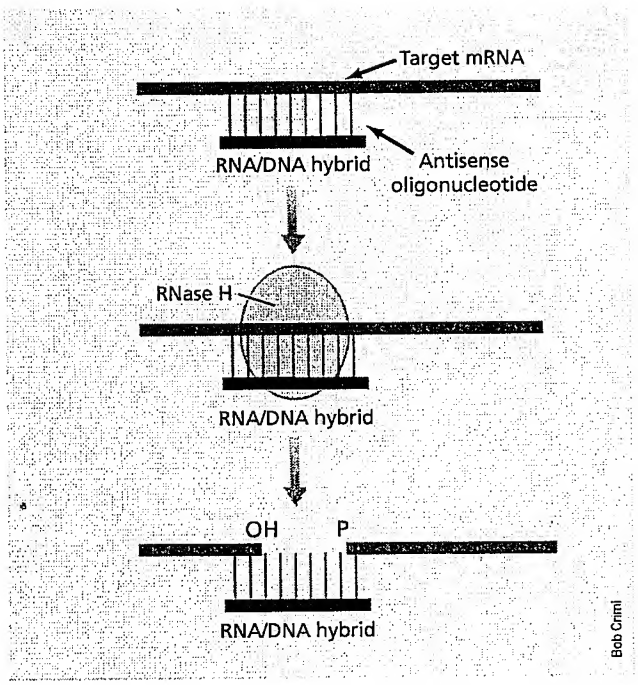

Figure 1 Model for antisense DNA ODN recruitment of RNAse H. Negatively charged ODNs interact with the target mRNA by Watson-Crick base pairing. The DNA-RNA hybrid is a substrate for endogenous RNAse H, which cleaves the RNA within the hybridized region, allowing the ODN to recycle.

automation of ODN synthesis in the early 1980s, it became relatively straightforward to obtain ODN s of any sequence and to test their ability to block gene expression via antisense base pairing.

Soon after the demonstration that phosphodiester backbone ODNs were effective as target-specific agents for blocking gene expression, several new backbone modifications were developed to improve the stability of the ODNs and to enhance their effectiveness. The most widely used modification is one in which the nonbridging oxygen is replaced by a sulfur atom, creating phosphorothioate ODNs[6]. This type of backbone formed the basis for the only Food and Drug Administration (FDA, Rockville, MD, USA)-approved antisense drug, Vitravene (Isis Pharmaceuticals, Carlsbad, CA, USA), which targets the cytomegalovirus *IE2* mRNA and is used to treat cytomegalovirus-associated retinitis. A second ODN, Genasense, which targets *Bcl2* (Genta, Berkely Heights, NJ, USA), has recently completed a phase III clinical trial for metastatic melanoma where it is being used in conjunction with standard chemotherapy, which the antisense potentiates. Several other phosphorothioate antisense ODNs are in earlier stages of clinical trials for a variety of cancers and inflammatory diseases.

The mechanisms of action of ODNs with respect to blocking gene function vary depending upon the backbone of the ODN[7–10]. Net negatively charged ODNs, such as phosphodiesters and phorphorothioates, elicit RNAse H–mediated cleavage of the target mRNA (Fig. 1). Other backbone modifications that do not recruit RNAse H, because of their lack of charge or the type of helix formed with the target RNA, can be classified as steric hindrance ODNs. Popularly used members of this latter group include morpholinos, 2′-*O*-methyls, 2′-*O*-allyls, locked nucleic acids and peptide nucleic acids (PNAs). These ODNs can block splicing, translation, nuclear-cytoplasmic transport and translation, among other inhibition targets. It is well beyond the scope of this article to delve further into the mechanisms of action of this diverse array of ODN modifications and for more detailed information, the reader is referred to specific reviews on this subject, which describe each of these modifications in detail[6,8].

Ribozymes

Ribozymes are RNA molecules that act as enzymes, even in the complete absence of proteins. They have the catalytic activity of breaking and/or forming covalent bonds with extraordinary specificity, thereby accelerating the spontaneous rates of targeted reactions by many orders of magnitude. The ability of RNA to serve as a catalyst was first shown for the self-splicing group I intron of *Tetrahymena thermophila* and the RNA moiety of RNAse P[11–13]. After the discovery of these two RNA enzymes, RNA-mediated catalysis has been found associated with the self-splicing group II introns of yeast, fungal and plant mitochondria (as well as chloroplasts)[14], single-stranded plant viroid and virusoid RNAs[15–17], hepatitis delta virus[18] and a satellite RNA from *Neurospora crassa* mitochondria[19]. Ribozymes occur naturally, but can also be artificially engineered for expression and targeting of specific sequences in *cis* (on the same nucleic acid strand) or *trans* (a noncovalently linked nucleic acid). New biochemical activities are being developed using *in vitro* selection protocols as well as generating new ribozyme motifs that act on substrates other than RNA[20].

The group I intron of *T. thermophila* was the first *cis*-cleaving ribozyme to be converted into a *trans*-reacting form, which we refer to as an intron/ribozyme[12,21], making it useful both in genomic research and as a possible therapeutic. In the *trans*-splicing reaction, a defective exon of a targeted mRNA can be exchanged for a correct exon that is covalently attached to the intron/ribozyme[22–24]. This occurs via a splicing reaction in which the exon attached to the intron is positioned by base pairing to the target mRNA so that it can be covalently joined to the 5′ end of the target transcript in a transesterification reaction. This reaction has been used to *trans*-splice wild-type sequences into sickle cell β-globin transcripts[25] and mutant *p53* transcripts[26] and replace the expanded triplets in the 3′-UTR of protein kinase transcripts in a myotonic dystrophy allele[27].

The endoribonuclease RNAse P is found in organisms throughout nature. This enzyme has RNA and one or more protein components depending upon the organism from which it is isolated. The RNA component from the *Escherichia coli* and *Bacillus subtilis* enzymes can act as a site-specific cleavage agent in the absence of the protein under certain salt and ionic conditions[28]. Studies of the substrate requirements for human and bacterial enzymes have shown that the minimal substrates for either enzyme resemble a segment of a transfer RNA molecule[29,30]. This structure can be mimicked by uniquely designed antisense RNAs, which pair to the target RNA, and serve as substrates for RNAse P–mediated, site-specific cleavage both in the test tube and in cells. It has also been shown that the antisense component can be covalently joined to the RNAse P RNA, thereby directing the enzyme only to the target RNA of interest[31]. Investigators have taken advantage of this property in the design of antisense RNAs, which pair with target mRNAs of interest to stimulate site-specific cleavage of the target[32] and for targeted inhibition of both herpes simplex virus and cytomegalovirus in cell culture[33–36].

A number of small plant pathogenic RNAs (viroids, satellite RNAs and virusoids), a transcript from a *N. crassa* mitochondrial DNA plasmid and the animal hepatitis delta virus undergo a self-cleavage reaction *in vitro* in the absence of protein. The reactions require neutral pH and Mg^{2+}. The self-cleavage reaction is an integral part of the *in vivo* rolling circle mechanism of replication. These self-cleaving RNAs

can be subdivided into groups depending on the sequence and secondary structure formed about the cleavage site. Small ribozymes have been derived from a motif found in single-stranded plant viroid and virusoid RNAs. On the basis of a shared secondary structure and a conserved set of nucleotides, the term 'hammerhead' has been given to one group of this self-cleavage domain[37,38] (Fig. 2a). The hammerhead ribozyme is composed of ~30 nucleotides. The simplicity of the hammerhead catalytic domain has made it a popular choice in the design of *trans*-acting ribozymes. Using Watson-Crick base pairing, the hammerhead ribozyme can be designed to cleave any target RNA. The requirements at the cleavage site are relatively simple, and virtually any UH sequence motif (where H is U, C or A) can be targeted.

A second plant-derived, self-cleavage motif, initially identified in the negative strand of the tobacco ringspot satellite RNA, has been termed the 'hairpin' or 'paperclip' (Fig. 2b)[17]. The hairpin ribozymes cleave RNA substrates in a reversible reaction that generates 2′,3′-cyclic phosphate and 5′-hydroxyl termini. Engineered versions of this catalytic motif also cleave and turn over multiple copies of a variety of targets in *trans*[39]. Substrate requirements for the hairpin include a GUC, with cleavage occurring immediately upstream of the G. The hairpin ribozyme also catalyzes a ligation reaction, although it is more frequently used for cleavage reactions.

There have been numerous applications of both hammerhead and hairpin ribozymes in cells for downregulating specific cellular and viral targets. Haseloff and Gerlach[40] designed a hammerhead motif in 1988 that can be engineered to cleave any target by modifying the arms that base pair with the target. Our laboratory first demonstrated that this hammerhead ribozyme motif had potential therapeutic applications was a study of cells engineered to express an anti-human immunodeficiency virus (HIV) *gag* ribozyme in which there was virtually complete inhibition of viral gene expression and replication[41]. Since this study, there have been literally thousands of applications of ribozymes targeting cellular and viral targets. A number of comprehensive reviews have been written that survey these applications, and the reader is referred to these for further treatment of this subject[42–46].

DNAzymes

A category of site-specific cleaving nucleic agents that has received considerable attention in the past several years is that of catalytic DNAs. Small DNAs capable of site specifically cleaving RNA targets have been developed via *in vitro* evolution (as no known DNA enzymes occur in nature)[47,48]. Two different catalytic motifs, with different cleavage site specificities, were found via this search. The most commonly used 10–20 enzymes (Fig. 2c) bind to their RNA substrates via Watson-Crick base pairing and site specifically cleave the target RNA, as do the hammerhead and hairpin ribozymes, resulting in 2′,3′-cyclic phosphate and 5′-OH termini. Cleavage of the target mRNAs results in their destruction and the DNAzymes recycle and cleave multiple substrates. Catalytic DNAs are relatively inexpensive to synthesize and have good catalytic properties[49–51], making them useful substitutes for either antisense DNA or ribozymes.

Several applications of DNAzymes in cell culture have been published including the inhibition of *veg F* mRNA and consequent prevention of angiogenesis[52], and inhibition of expression of the *bcr/abl* fusion transcript characteristic of chronic myelogenous leukemia[53]. A drawback of catalytic DNAs compared to ribozymes is that they can only be delivered exogenously, but they can be backbone-modified, perhaps allowing them to be delivered systemically in the absence of a carrier.

RNAi and siRNAs

RNAi refers to a group of related gene-silencing mechanisms sharing many common biochemical components in which the terminal effector molecule is a small 21–23-nucleotide antisense RNA. One mechanism uses a relatively long, dsRNA 'trigger,' which is processed by the cellular enzyme Dicer into short, 21–23-nucleotide dsRNAs, referred to as siRNAs (Fig. 3). The strand of the siRNA complementary to the target RNA becomes incorporated into a multi-protein complex termed the RNA-induced silencing complex (RISC), where it serves as a guide for endonucleolytic cleavage of the mRNA strand within the target site. This leads to degradation of the entire mRNA; the antisense siRNA can then be recycled[54]. In lower organisms, RNA-dependent RNA polymerase also uses the annealed guide siRNA as a primer, generating more dsRNA from the target, which serves in turn as a Dicer substrate, generating more siRNAs and amplifying the siRNA signal. This pathway is commonly used as a viral defense mechanism in plants.

The term siRNA is now generally used whenever the antisense

Table 1 Relative strengths and weaknesses of antisense technologies

Approach	Advantages	Disadvantages
Antisense ODNs	Can be modified to improve selectivity and efficacy Can be targeted to introns Easy to make	Can induce interferon (if long and has CpG) Can bind proteins (aptamer activity) Only exogenous delivery possible (synthetic) Off-target effects
Ribozymes	Can discriminate single base polymorphisms Can be used to correct defects Sequences can be appended to change target specificity Simple catalytic domain Can target introns/subcellular compartments	Requires GUC triplet—limits choice of target Binds proteins (aptamer activity)
DNAzymes	Inexpensive to make Good catalytic properties Can be modified for systemic delivery	Only exogenous activity Off-target effects?
RNAi	Effective at low concentrations Bypasses interferon pathway Can be delivered by multiple pathways Tissue-specific expression possible Nontoxic? Lasts longer?	Cannot target nuclear RNAs or introns No option for improving if target refractory Some reports of off-target effects

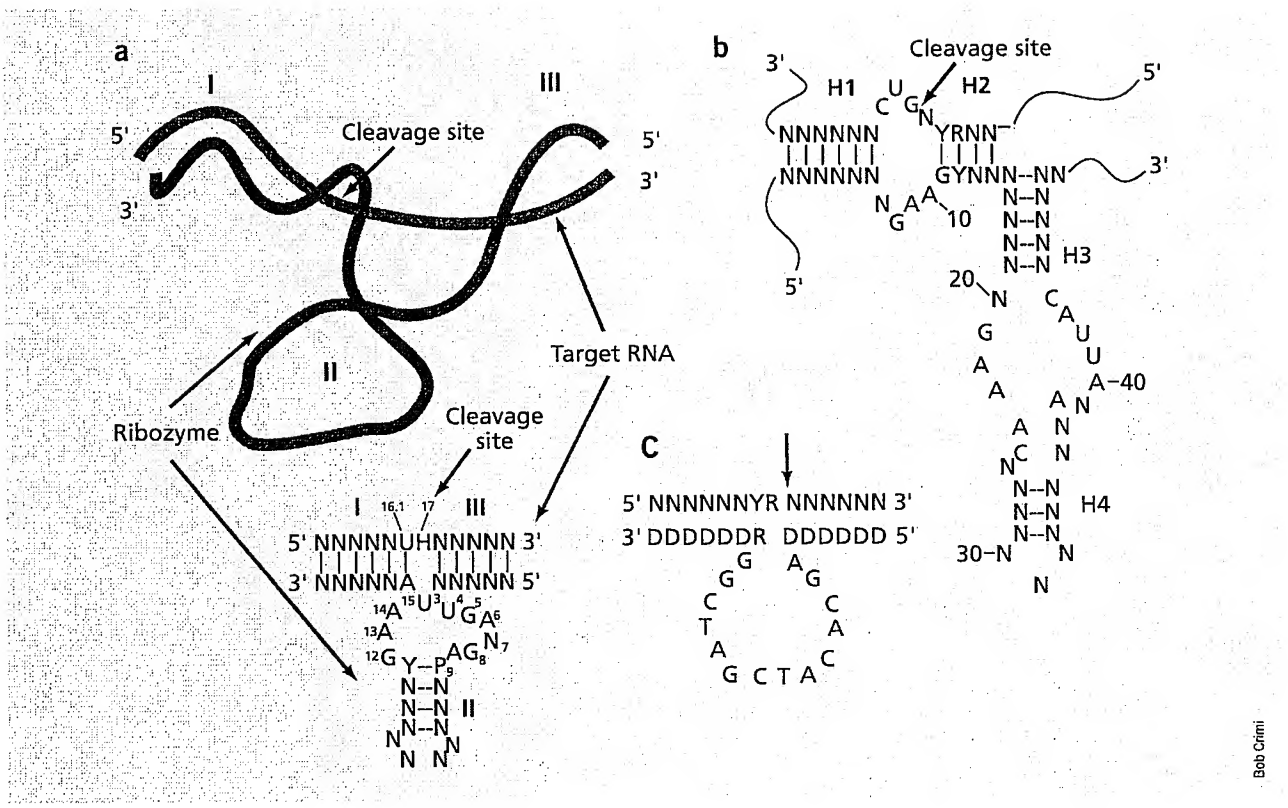

Figure 2 Generalized hammerhead ribozyme, hairpin ribozyme and DNAzyme motifs. (a) The hammerhead ribozyme in a *trans* configuration with a target RNA. The upper portion depicts an outline of the three-dimensional structure of the ribozyme bound to its substrate[98,99]. N represents A, C, G or U; H represents A, C or U. The numbering of the hammerhead ribozyme conforms to the standard convention adopted for this ribozyme[100]. The sites of cleavage are depicted by the arrows. (b) The hairpin ribozyme base-paired to a target RNA, using the same conventions as in a. (c) The 10–20 DNA enzyme[48]. The DNAzyme is depicted by D (for DNA) or A, G, C, T; Y, pyrimidine; and R, purine. The arrow depicts the site of cleavage. For further details, see text.

strand is completely complementary to the mRNA target site. The siRNA may consist of two separate, annealed single strands of 21 nucleotides, where the terminal two 3′-nucleotides are unpaired (3′ overhang). Alternatively, the siRNA may be in the form of a single stem-loop, often referred to as a short hairpin RNA (shRNA). Typically, but not always, the antisense strand of siRNAs is also completely complementary to the sense partner strand of the si/shRNA.

Recent experiments indicate that in fission yeast, dsRNA encoded by the centromeric DNA also mediates silencing of centromeric heterochromatin, and is dependent on components of the RNAi pathway[55,56]. Similar RNAi-like mechanisms are involved in silencing of the *Schizosaccharomyces pombe* mating type locus[57]. Chromatin silencing of an endogenous *ura4*$^+$ gene in *trans* is initiated by a *ura4*$^+$ long-stemmed (280 base pairs) hairpin encoded on an extra-chromosomal plasmid requiring both RNAi components and Clr4 (a histone methylase); spreading of heterochromatin through euchromatin requires the *S. pombe* ortholog of Swi6. Moreover, the same mechanism, using naturally occurring siRNAs derived from endogenous transposons, has been implicated in regulating normal host gene expression in *S. pombe* during meiosis[58].

In mammalian cells, long dsRNAs (usually greater than 30 nucleotides in length) trigger the interferon pathway, activating protein kinase R and 2′,5′-oligoadenylate synthetase[2]. Activation of the interferon pathway can lead to global downregulation of translation as well as global RNA degradation. However, shorter siRNAs exogenously introduced into mammalian cells have been reported to bypass the interferon pathway, although recent evidence suggests this may not always be the case[59].

The siRNA antisense product can also be derived from endogenous microRNAs. Data drawn from experiments in several paradigm systems, such as the *C. elegans* lin4/lin14 pathway, suggest the following pathway for microRNA biogenesis and gene regulation in animal cells. The ends of a transcript are removed in the nucleus by an exo III RNAse (Drosha, in human cells), forming a ~70 nucleotide pre-micro RNA fold-back intermediate[60]. Pre-microRNAs may be multicistronic, containing multiple hairpins directed against different target RNAs. The pre-microRNA is actively exported to the cytoplasm where Dicer processing trims the hairpin stem and removes the loop and sense strand to create the final 21–23-nucleotide antisense RNAi effector. In contrast to the prototypical si/shRNAs, the sense and antisense stem partner strands are not completely complementary, containing bubbles or bulges; both the structure and thermodynamic properties of the base pairing are critical for proper processing[61,62]. Moreover, the antisense strand contains mismatches to one or more sites in the 3′ untranslated region of the target mRNA, where binding mediates translational repression rather than mRNA degradation. MicroRNAs are widespread phylogenetically and conserved in some instances; they also exhibit temporal and spatial regulation[63]. A recent estimate for the

number of human microRNAs is 200–250 (ref. 64).

In human cells, experiments with siRNAs and microRNAs indicate that, regardless of the initial form or processing pathway, a final mature 21–23-nucleotide antisense RNA that is completely homologous to the mRNA will direct mRNA cleavage. In general, the effect of mismatches between siRNAs and target sites can vary from almost none to complete abrogation of activity, for reasons that are only partially understood; however, in at least one case, partial homology resulted in mRNA translation inhibition. In this report, an siRNAwith target mismatches designed to mimic a prototypical microRNA-target interaction mediated varying degrees of translational repression, depending on both the specific interaction and the number of target sites in the mRNA[65–67]. Consequently, it is likely that the structural features typical of siRNAs or microRNAs are important for processing and selection of the antisense strand in RISC and have important implications for the design of RNAi-inducing agents (see 'Target sequence' below).

RNAi can be activated by either exogenous delivery of preformed siRNAs or via promoter-based expression of siRNAs or shRNAs[68] (Fig. 4). Thus, RNAi has emerged as a potent mechanism to specifically knockdown mRNA transcripts to a few percent of their original levels by most methods of detection. RNAi appears to be more potent than antisense RNAs, ribozyme or RNAzymes for targeted message destruction, presumably because it exploits cellular machinery that efficiently directs the antisense component to the target mRNA for site-directed cleavage.

Comparative analyses

In the light of the above discussion, the question of what roles still exist for older technologies, such as antisense ODNs, ribozymes or DNAzymes versus RNAi is an important one. The various unique potential uses for each of the technologies are summarized below and in Table 1.

Targeting precursor RNA molecules. The majority of published evidence indicates that RNAi targets RNA molecules primarily in the cytoplasm in animal cells[69]. It is unclear at this time whether RNAi in mammals also affects chromatin organization and gene expression as it does in some lower eukaryotes and plants. RNAi-mediated chromatin silencing as found in yeast may also require the action of a group of clustered siRNAs in a localized area, as opposed to the one or a few siRNAs typically necessary for mRNA target downregulation. Short inhibitory RNAs have not been effective against intron target sites and may not be effective against RNAs that are exclusively nuclear, such as spliceosomal RNAs.

Ribozymes and antisense ODNs, on the other hand, can be designed to target introns and nuclear-localized RNAs. These agents may be more useful when it is necessary to selectively downregulate a sequence derived from a gene family of highly homologous sequences in which only the introns have grossly different sequences. By the same token, ribozymes can be used under circumstances where it is highly advantageous to degrade mRNA before it reaches the cytoplasm. For example, work in our laboratory has shown that an α-HIV ribozyme directed to the nucleolar compartment can successfully inhibit HIV replication[70]. Such selectivity in intracellular compartmentalization is not possible with antisense ODNs, DNAzymes or siRNAs.

For most sequences, though, cytoplasmic targeting is sufficient to achieve the desired downregulation, and here siRNAs can be the most effective reagents because of their reactivity at concentrations lower than those required for the same level of gene silencing mediated by the other agents[71,72]. (It should be pointed out, however, that the effective concentrations of RNAse H–dependent ODNs and siRNAs for a given target have been found to be equivalent in one study[72].) Thus far, there have been no direct comparisons between siRNAs and ribozymes or DNAzymes. Furthermore, as siRNAs can be produced by intracellular expression of siRNAs or shRNAs, it would be useful to compare expressed si/shRNAs with these other types of inhibitors.

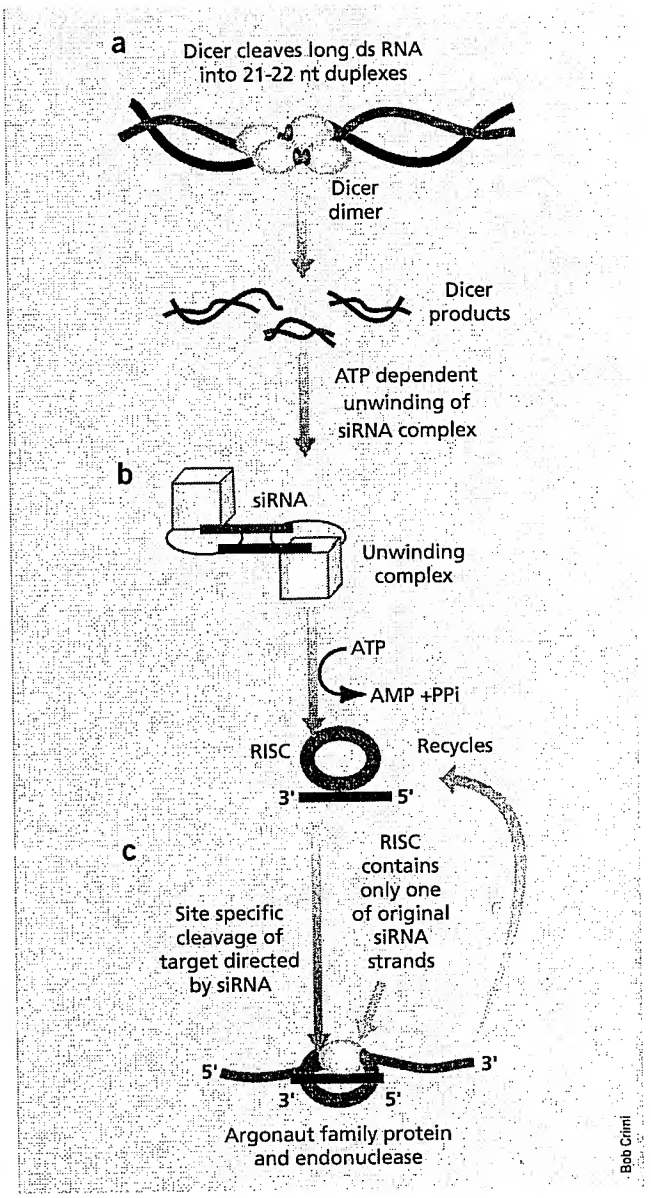

Figure 3 The RNAi pathway. (a) DsRNAs are cleaved by the enzyme Dicer into siRNAs. (b) The siRNAs are unwound before entry into RISC and the strand complementary to the target mRNA is incorporated into RISC. (c) RISC contains an endonuclease that cleaves only the target mRNA within the hybridized region.

Target sequence selection. Finding an effective target site within an mRNA can be problematic for antisense ODNs, ribozymes, DNAzymes and siRNAs. It is clear that there are sequences that are refractory to siRNAs[73,74] as well as to antisense ODNs and ribozymes[6,75]. The major limitation for each of these approaches is the

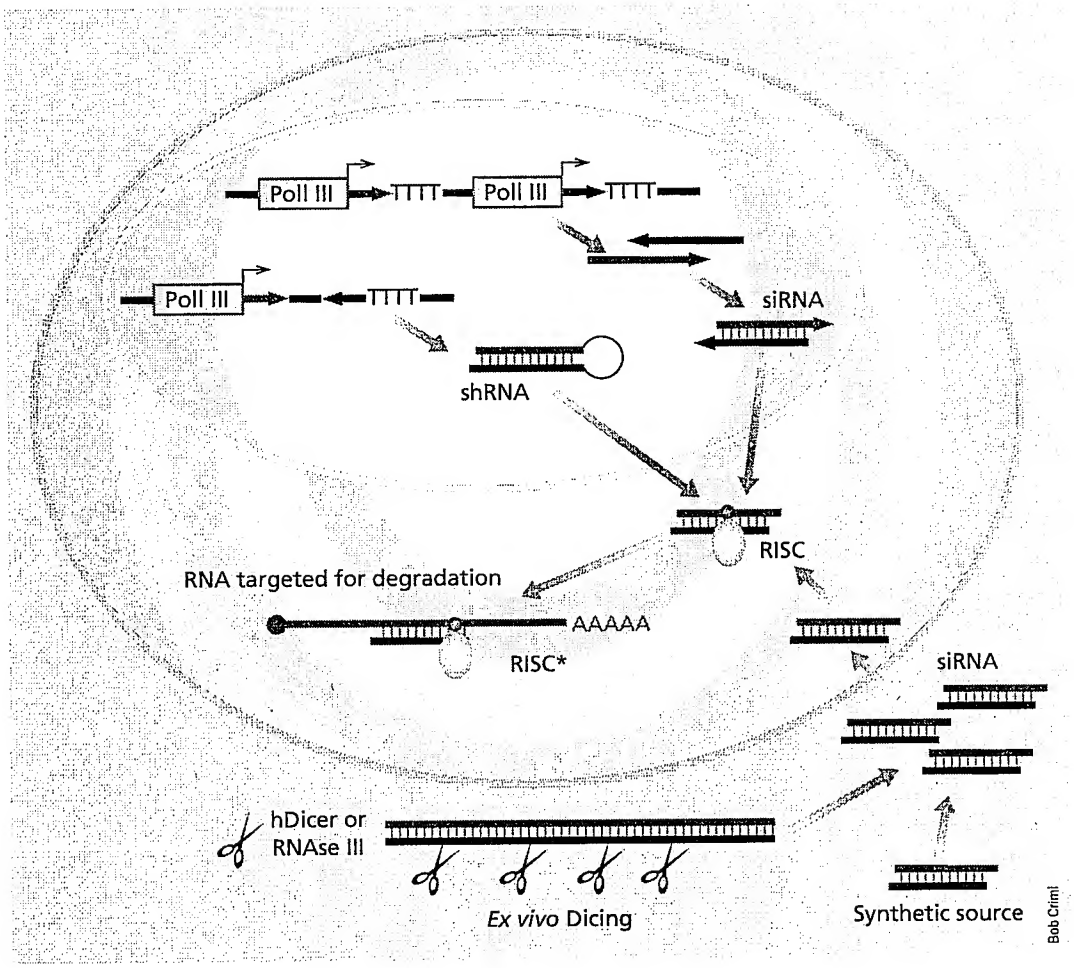

Figure 4 Various methods for introduction of siRNAs or shRNAs into mammalian cells. Small inhibitory RNAs can be generated from long dsRNAs *in vitro* via recombinant Dicer, they can be transcribed *in vitro* from plasmids or they can be chemically synthesized. These RNAs are transfected into cells using cationic lipid formulations. The expression cassettes depicted within the cell can create shRNAs or siRNAs as depicted. Both Pol II and Pol III systems have been used for intracellular promoter-based expression.

identification of a sequence/antisense combination that provides the most potent knockdown at the lowest possible concentration of antisense agent. The mere predicted folding pattern of a target mRNA is usually not sufficient to find such a combination. To date, target site selection for all of these approaches is best done by systematically testing a variety of potential targets[72] although there are several commercial and academic websites that have algorithms for identifying accessible target sequences. However, recent publications suggest that the thermodynamic properties of the sense/antisense base pairing in the siRNA are very important in the choice of target sequence, and may explain the weak suppression by some previously tested siRNAs[61,76].

If the choice of accessible target sites is limited, use of a ribozyme may not be possible if the site does not contain an appropriate triplet cleavage site—a limitation not shared by antisense ODNs or siRNA design. In addition, if a specific target site is refractive to siRNA, there are currently no options for improving cleavage of that site. A number of colocalization options exist to improve ribozyme accessibility by direction to specific cell compartments and (sequence-directed) colocalization with the target[71,77–80]. The size requirement of siRNAs and the need to get cytoplasmic accumulation restricts the use of appended sequences, although there are exceptions[81], and more may emerge as the biochemical processing pathways of siRNAs and the related microRNAs are better understood. Hybrid RNAs sharing both microRNA and siRNA characteristics could be used for processing multiple siRNAs and/or microRNAs from a single transcript, possibly providing multiple targeting for treating genetically variable viruses, such as HIV and hepatitis C virus.

Off-target effects. In choosing a method for targeted knockdown of gene expression, an important consideration has to be the potential for off-target, non-sequence specific effects. Each of the respective methods (antisense ODNs, ribozyme and siRNA) has the potential for such effects. For instance, antisense ODNs have been shown to direct RNAse H cleavage of nontargeted RNAs by virtue of the fact that only six or seven contiguous base pairs with the target RNA are required to direct cleavage[82]. In contrast, ribozymes are much more sensitive to polymorphisms at the cleavage site (though relatively less so in the hybridizing arms, depending upon position) and have therefore been used for discriminating between single nucleotide polymorphisms[83–85]. There is at least one report in the literature of a multitude of off-target effects by synthetic siRNAs[86], although a separate study has suggested that siRNAs were highly specific[87]. These two studies differed in the choice of targets for siRNA inhibition. In the former, the targets were endogenous transcripts, whereas in the latter a reporter construct was the target. It is too soon to draw strong conclusions about the generality of off-targeting by siRNAs, but clearly this potential problem needs to be further addressed by investigators in the field.

For each of the antisense reagents discussed in this review, the off-

target effects may be a consequence of the levels of the agent delivered to cells. As an example, a standard tissue culture experiment might use ~5×10^5 cells in 2.0 ml of medium. If one applies a 10 nM concentration of a single strand of an antisense agent to these wells, roughly10^7 molecules of the antisense agent per cell is present. If the uptake is only 1%, there will still be 10^5 molecules per cell.

For siRNA, it is critical to keep the concentration as low as possible, because the double-stranded molecules can yield two strands capable of eliciting off-target effects. An additional concern for siRNAs is that the capacity of RISC for interaction with transfected or expressed siRNAs is unknown. It is highly unlikely that all the RNA entering the cytoplasm will be incorporated into RISC or be immediately degraded, and therefore it will be available for other cellular processes.

High concentrations of the other antisense agents is risky because ODNs and ribozymes act by diffusing to their targets, a process in which they could encounter many partially homologous sequences in the process. The lesson here is that all of these agents must be used at the lowest effective concentration to minimize unwanted side activities.

Side effects. Other potential issues of importance in comparing approaches for sequence-specific knockdown of mRNAs are 'unanticipated side effects.' For instance, CpG motifs in antisense DNA ODNs elicit strong innate and acquired immune responses *in vivo*, most likely via interactions with Toll-like receptor[88,89]. Both antisense ODNs and ribozymes could function as aptamers, binding proteins that are unrelated to the targets of these ODNs[90]. Finally, at least two recent reports suggest that siRNAs and shRNAs can activate arms of the interferon response pathways, which could lead to nonspecific inhibition of protein synthesis and global RNA degradation[59,91]. As powerful as all three of these target knockdown technologies can be, there is always the concern that one can be led astray by such side effects. Again, there is a strong requirement for further experimentation to sort out which of these agents is most likely to generate unwanted side effects.

Duration of effect. Another consideration in choosing the 'most efficacious' antisense agent is whether the application requires long-term or short-term knockdown of the target molecule. Applications involving chronic infections, such as HIV and hepatitis C virus, recalcitrant cancers, some dominant or codominant genetic abnormalities and generation of knockdown animals, to name a few, will require continuous application or expression of the antisense agent. Moreover, if endogenous expression is the best route for application, then ribozyme, RNAi and antisense RNA are the best approaches as these RNAs can be expressed from vector backbones, whereas synthetic ODNs and DNAzymes can only be delivered exogenously. Ribozymes, RNAi and antisense RNAs can also be expressed using inducible or tissue-specific promoter systems, making controlled expression possible[75,92,93]. For transient applications, each of these approaches can be applied, provided that the desired cellular delivery can be achieved. The use of backbone modifications that enhance the serum/cellular half-lives of antisense ODNs, ribozymes, DNAzymes and siRNAs makes each of these reagents useful for short-term inhibition of gene expression[6,94]. The efficiency of delivery will continue to be the limiting factor for stabilized antisense compounds. Delivery is a key concern if the antisense agents are going to be used in a therapeutic setting. To date, there is no single reagent or backbone modification that can be effectively used for all the different antisense agents.

Conclusions

As the biochemical mechanisms of RNAi become better understood, the use of siRNA will continue to expand; however, RNAi is unlikely to supplant the use of antisense, ribozymes, DNAzymes and related approaches for many applications. In practice, the choice of antisense methodology will depend on the specific circumstances of the application. Moreover, the addition of new technologies to the antisense toolbox is expected to increase the range of applications and allow fine-tuning of the general approach. One recent example is inhibition by RNAu, where a mutated U1 small nuclear RNA is attached to a 10-nucleotide antisense sequence targeted against a site in an mRNA terminal exon. In this case, the inhibitory RNA prevented polyA addition and triggered mRNA degradation[95].

Regardless of which antisense technology is applied, the challenge of ensuring specificity remains paramount because of the potential for nontargeted alteration of gene expression. One perceived, but not yet demonstrated, advantage of RNAi is that it takes advantage of cellular machinery, RISC, specifically designed for selective inhibition of targeted transcript expression. However, one potential side effect is that the mechanism may be prone to saturation, thereby leaving unprocessed small duplexes of RNAs free to enter into other cellular pathways. Thus, it is imperative to identify target sequences that are effectively downregulated at low nanomolar or even sub-nanomolar concentrations of siRNA. The same qualification holds for the other antisense-based mechanisms. For each of these approaches it would also be in the best interest of investigators to verify the phenotype with a second or third agent directed at a different sequence in the same transcript. For siRNAs, there is always the potential that even low concentrations can result in off-target effects via participation in the microRNA pathway or perhaps even at the level of chromatin remodeling[66,96,97].

Alternatively, siRNAs provide an additional antisense-based tool that may be even more powerful combined with the other nucleic acid–based therapies. The discovery of RNAi has certainly accelerated the pace at which targeted post-transcriptional gene silencing is being applied as a tool for identifying gene function and as a therapeutic agent. It remains to be determined whether the recent concerns about off-target effects and interferon pathway induction become a roadblock or merely a detour.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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APPENDIX

C

The promises and pitfalls of RNA-interference-based therapeutics

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The discovery that gene expression can be controlled by the Watson–Crick base-pairing of small RNAs with messenger RNAs containing complementary sequence — a process known as RNA interference — has markedly advanced our understanding of eukaryotic gene regulation and function. The ability of short RNA sequences to modulate gene expression has provided a powerful tool with which to study gene function and is set to revolutionize the treatment of disease. Remarkably, despite being just one decade from its discovery, the phenomenon is already being used therapeutically in human clinical trials, and biotechnology companies that focus on RNA-interference-based therapeutics are already publicly traded.

Before 1980, RNA was generally considered to be no more than a passive intermediate carrying information between DNA and protein synthesis. The discovery of catalytic RNAs in the early 1980s merited a shared Nobel prize to Tom Cech and Sidney Altman, and in 1986 the concept of 'the RNA world', an idiom created by Walter Gilbert, was proposed. Today, this is a common expression, and RNA has claimed a pivotal place in cellular biology.

Just ten years ago, RNA's functional repertoire was expanded further with the discovery in the nematode *Caenorhabditis elegans*[1] that double-stranded RNAs (dsRNAs) can trigger silencing of complementary messenger RNA sequences, and the term 'RNA interference' (RNAi) was born. Shortly thereafter, short dsRNAs — or short interfering RNAs (siRNAs) (reviewed in ref. 1) — were generated artificially and used to demonstrate that this process also occurs in mammalian cells, usually, but not always, without triggering the innate immune system, which normally recognizes RNAs as part of an antiviral defence mechanism (see page 421). The knowledge that small RNAs can affect gene expression has had a tremendous impact on basic and applied research, and RNAi is currently one of the most promising new approaches for disease therapy.

That RNAi could be triggered *in vivo* in mammals was first shown in animals infected with hepatitis B virus[2]. This was followed by the first therapeutic application of siRNAs: siRNAs were targeted to *Fas* mRNA in a mouse model of autoimmune hepatitis, resulting in protection of the treated animals against liver fibrosis[3]. In 2004, only six years after the discovery of RNAi, the first siRNA-based human therapeutics — developed as treatments for wet age-related macular degeneration — entered phase I clinical trials. RNAi is one of the fastest advancing fields in biology, and the flow of discoveries gives true meaning to the expression 'from the bench to the bedside'.

Although much is known about the mechanisms of RNAi, there are a number of challenges that applications of this gene-silencing technology need to overcome. For one, RNAi is a fundamentally important regulatory mechanism in the cell, and tapping into it in the interests of therapeutic benefit could result in side effects. Exogenously introduced dsRNA sequences can sequester components that make up the cellular machinery involved in gene silencing (see page 396), thereby reducing the accessibility of the machinery to a class of small RNAs known as microRNAs (miRNAs) that are entering the natural cellular pathway[4,5]. In addition, some synthetic siRNAs contain sequence motifs that can induce type I interferon responses and stimulate the production of pro-inflammatory cytokines[6–8].

During the past few years, many scientists have searched for solutions to overcome these limitations and to increase the safety of potential RNAi-based therapeutics. This article explores recent strategies to minimize undesirable secondary effects, describes new approaches to delivery and discusses RNAi therapies that are being tested. As it is anticipated that this technology will be applied to an increasing range of diseases, the potential problems and solutions that could one day transform RNAi into a conventional treatment for human diseases warrant careful attention.

Endogenous gene silencing

The effector RNA molecules of RNAi consist of ~20–30 nucleotides[9]. They are complexed with the protein components of the RNA-induced silencing complex (RISC). Its catalytic core in plants and animals (with the exception of single-celled organisms) is AGO2, a member of the highly conserved Argonaute protein family[10]. These small RNAs can silence gene expression by two mechanisms: post-transcriptional gene silencing (PTGS)[11], and transcriptional gene silencing (TGS)[12,13] (Fig. 1). PTGS can, in turn, be divided into two main mechanisms: direct sequence-specific cleavage, and translational repression and RNA degradation. Direct sequence-specific cleavage occurs when the targeted mRNA is perfectly complementary to the siRNA and is degraded after site-specific cleavage by the RISC. Translational repression and RNA degradation occur when the small RNA guide sequence has only limited complementarity to the target in the 'seed' region (nucleotides 2 to 8 from the 5′ end of the guide strand), with base-pairing usually occurring in the 3′ untranslated region (UTR). The latter mechanism is used by miRNAs.

TGS has been demonstrated in *Schizosaccharomyces pombe* (fission yeast), plants and, most recently, mammalian cells[14–17]. In *S. pombe*, the process is mediated by the RNA-induced transcriptional silencing complex (RITS), which contains Ago1, the chromodomain protein Chp1 and the glycine and tryptophan (GW)-repeat-containing protein Tas3 (ref. 18) (see page 413). Although in mammalian cells the mechanism by which small-RNA-directed silencing occurs is still hotly debated, both AGO1 and AGO2 have been shown to be integral to the overall

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process[19,20]. Most recently, a miRNA (miR-320) has been shown to regulate transcription of the POLR3D subunit of RNA polymerase III (Pol III)[21] in human cell culture.

Endogenous small RNAs have been found in various organisms, including humans, mice, the fruitfly *Drosophila melanogaster* and *C. elegans*. Many of these originate from transposons, viruses and repetitive sequences and are characterized by their interactions with the PIWI subfamily (or PIWI clade) of Argonaute proteins[22–25] — these are thus named PIWI-interacting RNAs (piRNAs). The identification of piRNAs has been restricted to germline cells. Recently, a new class of endogenous siRNAs (endo-siRNAs or esiRNAs) has been identified in the gonads and somatic tissues of *D. melanogaster*[26–29] and in mouse oocytes[30,31]. In mice, endo-siRNAs have been proposed to regulate retrotransposon movement[30,31]. Several families of small RNAs, including repeat-associated siRNAs (ra-siRNAs), tiny non-coding RNAs (tncRNAs), *trans*-acting siRNAs (ta-siRNAs) and scan RNAs (scnRNAs) (Table 1) are found in fungi, plants and animals, but so far none of these has been observed in mammals. The evidence suggests that piRNAs act through different cellular pathways from siRNAs and miRNAs and so could offer alternative targeting strategies for therapeutic targets.

Superior designs for small molecules

Cellular genes can be targeted by exogenous introduction of siRNAs, which then take advantage of the endogenous PTGS mechanism. The siRNAs can be either transfected into cells, where they enter the RISC directly, or generated within cells through gene expression by the use of vectors containing Pol II or Pol III promoters. These RNAi triggers can be expressed in animals and plants, but not in *S. pombe*, in the form of miRNAs or as short hairpin RNAs (shRNAs), which are cleaved into small (~21–25-nucleotide) RNAs by the enzymes Drosha and/or Dicer. In both cases, if the two strands of the RNA trigger are completely complementary, the passenger strand is cleaved by AGO2 (refs 32, 33), leaving behind a single-stranded guide sequence, which acts as the template for recognition of the targeted gene sequence by the RISC (Fig. 1).

Most of the impending therapeutic applications based on RNAi propose using direct introduction of synthetic siRNAs. The advantage of

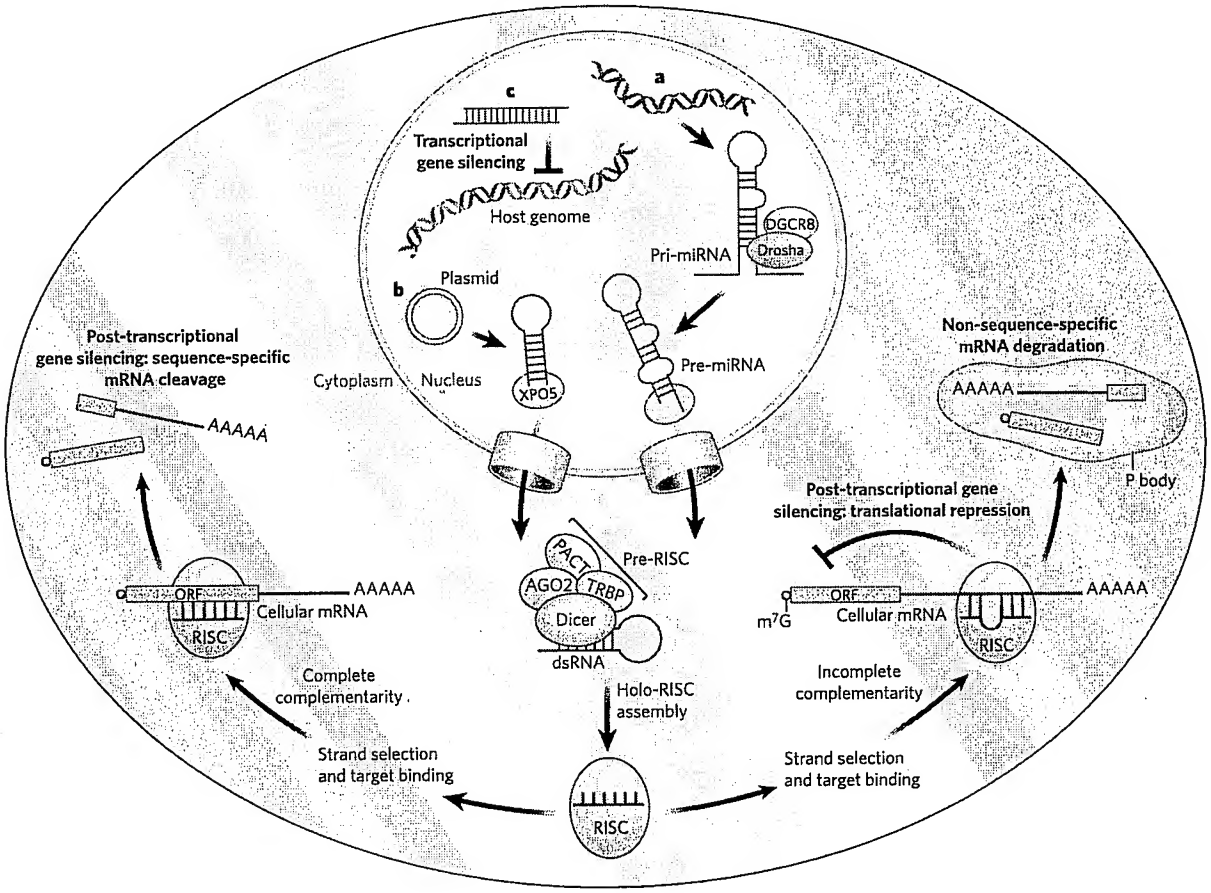

Figure 1 | Mechanisms of cellular gene silencing. a, Primary microRNAs (pri-miRNAs) are, in plants and animals, processed by Drosha and its partner DGCR8 into precursor miRNAs (pre-miRNAs) and then transported to the cytoplasm by exportin 5 (XPO5). In the cytoplasm, they are bound by a Dicer-containing pre-RISC and processed to yield the guide sequence that is loaded into the holo-RISC, which contains all the components required for gene silencing. AGO2 is the catalytic core of the RISC (present but not shown in the schematically drawn holo-RISC). The guide sequence binds to the corresponding target sequences in the 3′ UTRs of cellular mRNAs. If the miRNA guide sequence is fully complementary to its target site (left pathway), it triggers site-specific cleavage and degradation of the mRNA through the catalytic domain of AGO2. If the base-pairing is incomplete (right pathway) but includes pairing of the seed region (nucleotides 2–8 of the miRNA) with the target, translational inhibition occurs, and this can be accompanied by non-sequence-specific degradation of the mRNA in P bodies. **b**, Similarly to miRNAs, artificially transcribed shRNAs (in this case from a plasmid) are transported to the cytoplasm by XPO5. The dsRNA in the cytoplasm is recognized and processed by Dicer into ~21–25-nucleotide siRNA fragments that are loaded into the RISC. The siRNAs can target complementary sequences of cellular mRNAs and trigger their degradation through AGO2-mediated cleavage. **c**, When siRNAs are present in the nucleus and are complementary to promoter regions, they can trigger chromatin remodelling and histone modifications that result in transcriptional gene silencing. In mammalian cells, the details of this mechanism are still under investigation but are known to include Argonaute-family proteins. Accessory proteins indicated in the figure are TRBP (HIV *tar*-RNA-binding protein; also known as TRBP2P) and PACT (activator of protein kinase PKR; also known as PRKRA). m^7G, 7-methylguanosine.

Table 1 | Cellular small RNAs involved in gene silencing

Class	Size (nucleotides)	Functions	Mechanisms	Origin	Organisms found in
siRNAs	21–25	Regulating gene expression, providing antiviral response, restricting transposons	RNA degradation, transposon restriction	Intergenic regions, exons, introns	*Caenorhabditis elegans*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Oryza sativa* (rice)
endo-siRNAs	21–25	Restricting transposons, regulating mRNAs and heterochromatin	RNA degradation	Transposable elements, pseudogenes	*D. melanogaster*, mammals
miRNAs	21–25	Regulating gene expression at the post-transcriptional level	Blocking translation, RNA degradation	Intergenic regions, introns	*C. elegans*, *D. melanogaster*, *S. pombe*, *A. thaliana*, *O. sativa*, mammals
piRNAs	24–31*	Regulating germline development and integrity, silencing selfish DNA	Unknown	Defective transposon sequences and other repeats	*C. elegans*, *D. melanogaster*, *Danio rerio*, mammals
ra-siRNAs	23–28	Remodelling chromatin, transcriptional gene silencing	Unknown	Repeated sequence elements (subset of piRNAs)	*C. elegans*, *D. melanogaster*, *S. pombe*, *Trypanosoma brucei*, *D. rerio*, *A. thaliana*
ta-siRNAs	21–22	*Trans*-acting cleavage of endogenous mRNAs	RNA degradation	Non-coding endogenous transcripts	*D. melanogaster*, *S. pombe*, *A. thaliana*, *O. sativa*
natRNAs	21–22	Regulating gene expression at the post-transcriptional level	RNA degradation	Convergent partly overlapping transcripts	*A. thaliana*
scnRNAs	26–30	Regulating chromatin structure	DNA elimination	Meiotic micronuclei	*Tetrahymena thermophila*, *Paramecium tetraurelia*
tncRNAs	22	Unknown	Unknown	Non-coding regions	*C. elegans*

*C. *elegans* piRNAs are 21 nucleotides. endo-siRNAs, endogenous siRNAs; miRNAs, microRNAs; natRNAs, natural antisense transcript siRNAs; piRNAs, PIWI-interacting RNAs; ra-siRNAs, repeat-associated siRNAs; scnRNAs, scan RNAs; siRNAs, short interfering RNAs; ta-siRNAs, *trans*-acting siRNAs; tncRNAs, tiny non-coding RNAs.

using a chemically synthesized molecule is that chemical modifications can be introduced to increase stability, promote efficacy, block binding to unintended targets that contain sequence mismatches (specific off-target effects), and reduce or abrogate potential immunostimulatory effects (general off-target effects). However, the effects of these molecules are transient, whereas the promoter-expressed shRNAs or miRNAs can potentially mediate long-term silencing with a single application.

Conventional siRNAs are ~22 nucleotides and have 3′ dinucleotide overhangs that mimic Dicer cleavage products. Because not all siRNAs achieve equivalent levels of target knockdown, large-scale siRNA screening is often performed for any given target to find the most potent inhibitors. These have yielded some rules for siRNA design. For example, to facilitate incorporation into the RISC, the 5′ end of the antisense (guide) strand should be designed to have a lower thermodynamic stability than the 5′ end of the sense strand. The proportion of the nucleotides guanosine and cytidine should be around 50% or lower, and targeting of known protein-binding sites in mRNA regulatory regions should be avoided because binding of regulatory proteins may block siRNA–target pairing. For the same reason, intramolecular structures in the target should be avoided. Statistical analyses have also found a preference for certain nucleotides at specific positions within the siRNA[34]. Many computer programs are available for identifying the optimal target sequences for a given gene[34,35]. One of these, an artificial neural network, has been used to develop a genome-wide siRNA library for humans and to identify effective siRNAs for 34 targets[36].

Chemical modifications are often included in the design of synthetic siRNAs. Selective addition of phosphorothioate linkages or substitution of 2′ fluoropyrimidines or a 2′-*O*-methyl for the 2′ ribose at certain positions does not compromise siRNA activity and concomitantly increases resistance to ribonucleases[37], which is important for *in vivo* applications. A single 2′-*O*-methyl group on the passenger strand of an siRNA duplex can abrogate activation of the Toll-like receptors[38] and prevent toxicities due to the activation of type I interferon pathway gene expression. It has recently been demonstrated that fluoro-β-D-arabinonucleic acid (FANA[39] or as 4′-*S*-FANA[40]) or arabinonucleic acid (ANA[41]) modifications can increase both the serum stability and the potency of siRNAs. Some chemical modifications also have the important advantage of decreasing or blocking the activity of the siRNA's sense (passenger) strand, thereby reducing specific off-target effects. Other modifications, such as the addition of lauric acid, lithocholic acids and cholesterol derivatives, can be made to increase cellular uptake[42], which is currently one of the main hurdles of RNAi therapy.

Breaking and entering

Therapeutic applications of siRNAs require effective delivery to the target cells and tissues. The two main strategies are delivery of chemically synthesized siRNAs (non-viral delivery), or delivery of shRNA-encoding genes by engineered viruses that will ultimately generate siRNAs by transcription in the target cells.

Non-viral delivery

Because of their size and negative charge, siRNAs cannot easily cross cell membranes. Delivery has therefore been one of the major challenges for RNAi technology. Various means of delivery have been developed and tested in murine and non-human primate models, ranging from the injection of naked RNAs into a target organ such as the lung or eye to systemic delivery of the RNA in nanoparticles, complexed with polycations, attached to cholesterol groups or conjugated with cell-surface receptors. Some delivery approaches are detailed in Fig. 2.

Two polymers that have been examined for their delivery properties are atelocollagen and chitosan. Chitosans have mucoadhesive properties and have been used for intranasal delivery to bronchiolar epithelial cells[43]. Intranasal delivery has proved an effective means of delivering siRNA in mice[44] and in non-human primates[45] to block respiratory syncytial virus infection of the upper respiratory tract. In fact, the delivery of siRNAs to mucosal membranes seems to be an effective approach in general. For example, intravaginal delivery of lipid-encapsulated siRNAs targeting herpes simplex virus 2 (HSV-2) provided protection against lethal viral infection in more than two-thirds of the siRNA-treated mice[46].

Targeting of anti-apolipoprotein B (APOB) and peroxisome proliferator-activated receptor-α (PPAR-α) siRNAs to the liver has been achieved by means of a 'membrane-active' polymer, which can mask its activity until it reaches the endosome, resulting in the delivery of siRNAs to hepatocytes after a simple intravenous injection[47]. A different siRNA delivery approach used transferrin conjugated to a cyclodextrin-polycation polymer to deliver siRNAs targeting the Ewing's sarcoma *Ews–Fli1* fusion mRNA by means of the transferrin receptor in mice[48], resulting in inhibition of tumour progression. And conjugation of an siRNA to a cholesterol group permitted its delivery to the liver and the jejunum, where it downregulated its target, APOB, leading to consequent lowering of blood cholesterol levels in a murine model system[49].

An important advance for siRNA delivery was the successful application of stable nucleic-acid lipid particles decorated with polyethylene glycol (PEG) polymer chains (termed SNALPs) for the delivery of siRNAs directed against *APOB* mRNA (*APOB*-targeted siRNAs) to the livers of

non-human primates[50]. In this case, the siRNA effect of a single intravenous injection lasted for more than 11 days and resulted in greater than 90% target knockdown and no toxicity[50]. These exciting results have increased confidence in the potential of therapeutic siRNAs for treating liver diseases.

Until recently, most approaches to *in vivo* delivery have targeted a particular organ, primarily the eye, the lungs or the liver. A significant advance in targeting siRNAs to a specific class of leukocytes involved in gut inflammation has now been reported[51]. In this study, a cyclin D1 (*Cyd1*)-targeted siRNA was loaded into stabilized nanoparticles, the surfaces of which incorporated an antibody specific for a receptor expressed by the leukocytes. The targeted siRNA-containing nanoparticles downregulated the cyclin D1 target, suppressed leukocyte proliferation and reversed experimentally induced colitis in mice[51].

Delivery of siRNAs to the nervous system has been particularly challenging. The brain is notoriously refractory to targeting because of difficulties in crossing the blood–brain barrier. However, delivery of siRNAs to the peripheral nervous system by direct infusion into the brain for the relief of chronic pain[52–54] or anxiety[55] has been demonstrated in rats. Conjugates of liposomes and antibodies or neuropeptides have also been used to deliver siRNAs into the murine brain[56]. Nevertheless, these methods do not target neurons, and a less invasive alternative to direct cranial injection is required to make such therapies more palatable.

A recent study unlocked the possibility of selective delivery of siRNAs to the central nervous system by systemic intravenous injection[57]. The siRNA involved — designed to target Japanese encephalitis virus — was conjugated with a short peptide derived from the rabies virus glycoprotein, which binds to the neuronal cell acetylcholine receptor. After transvascular delivery, 80% of the mice treated with the therapeutic siRNA survived infection with Japanese encephalitis virus, whereas 100% of the untreated controls died from complications of the infection[57].

Another interesting approach that allows systemic and targeted siRNA delivery uses a protamine–antibody fusion protein[58]. The protamine moiety is linked to the heavy-chain antigen-binding region (Fab) of an antibody to the human immunodeficiency virus 1 (HIV-1) envelope protein gp160. The positively charged protamine binds the negatively charged siRNAs — which are targeted against the HIV gene *gag* — allowing selective delivery to cells expressing the gp160 envelope protein on their surfaces[58]. This results in internalization of the antibody–siRNA complex, release of the siRNAs and downregulation of the HIV Gag-encoding

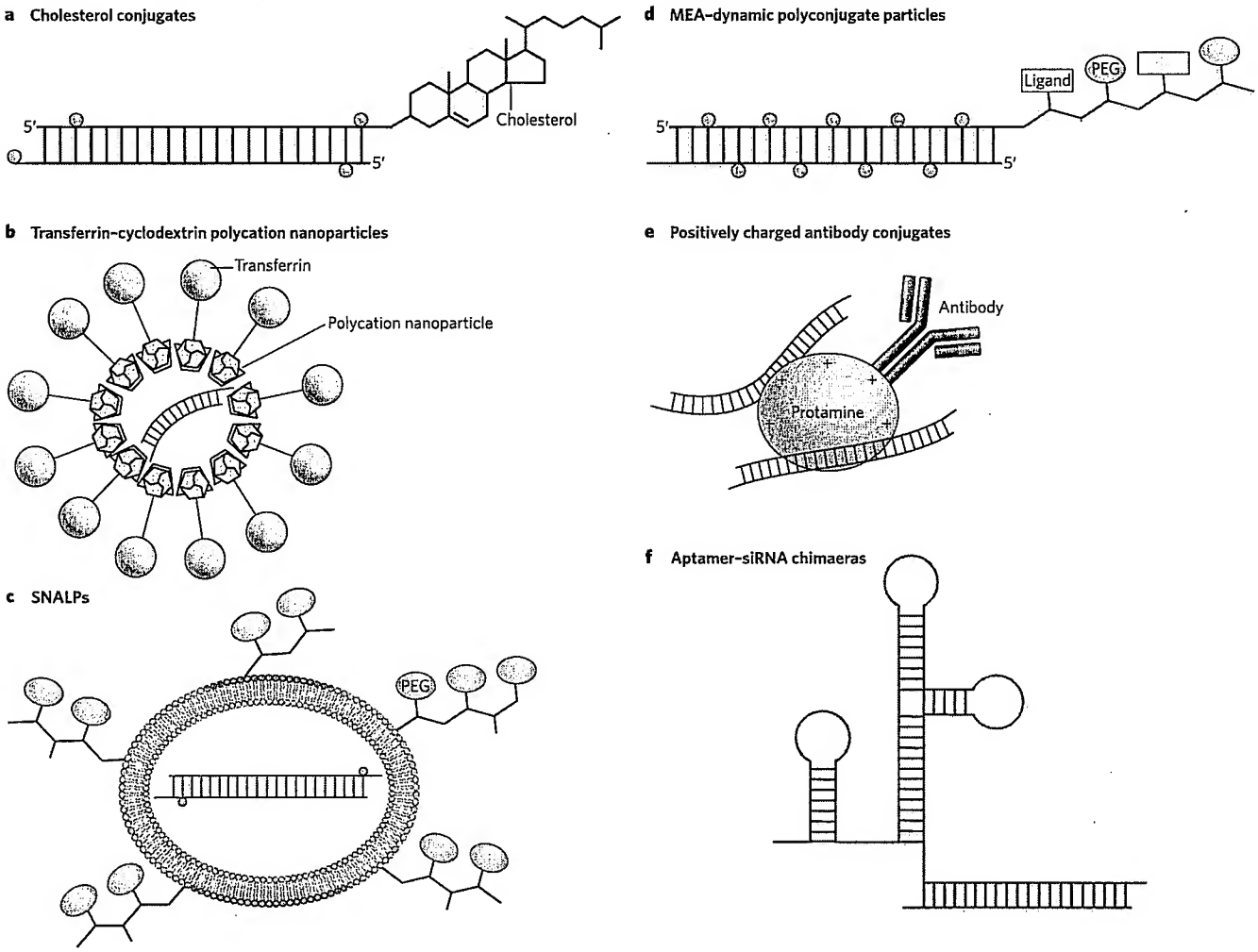

Figure 2 | *In vivo* delivery strategies for therapeutic siRNAs. **a**, Cholesterol groups can be linked to modified siRNAs to enhance their stability before systemic delivery. The most common siRNA modifications are 2′-*O*-methyluridine or 2′-fluorouridine substitutions (blue circles) combined with phosphorothioate linkages. **b**, Polycation nanoparticles can direct delivery of the siRNAs to specific cells through the use of surface ligands (such as transferrin) that bind to receptors on target cells. **c**, SNALPs encapsulate modified siRNAs into cationic or neutral lipid bilayers coated with diffusible PEG–lipid conjugates. SNALPs allow siRNAs to be taken up by cells and released by endosomes. **d**, Masked endosomolytic agent (MEA)–Dynamic PolyConjugates (DPCs) are similar to SNALPS but smaller, and contain a ligand that allows targeted cell delivery. The release of the siRNA from the endosome is also improved by the inclusion of a pH-labile bond in the MEA–DPC particles. **e**, Tagging specific antibodies with protamine or other positive charges allows the delivery of siRNAs to specific cell types via receptor-mediated uptake. **f**, Chemically linking or co-transcribing siRNAs with RNA aptamers allows the targeted delivery of the siRNAs to cells expressing the appropriate receptor.

Table 2 | Current clinical trials of RNAi-based therapeutics

siRNA	Company	Disease	Stage
Bevasiranib	Acuity Pharmaceuticals	Wet age-related macular degeneration	Phase III
		Diabetic macular oedema	Phase II
Sirna-027	Merck-Sirna Therapeutics	Wet age-related macular degeneration	Phase II
RTP801i-14	Quark Pharmaceuticals, and Silence Therapeutics	Wet age-related macular degeneration	Phase I/IIA
ALN-RSV01	Alnylam Pharmaceuticals	Respiratory syncytial virus infection	Phase II
NUC B1000	Nucleonics	Hepatitis B	Phase I
Anti-*tat*/*rev* shRNA	City of Hope National Medical Center, and Benitec	AIDS	Pilot feasibility study
CALAA-01	Calando Pharmaceuticals	Solid tumours	Phase I
TD101	TransDerm, and the International Pachyonychia Congenita Consortium	Pachyonychia congenita	Phase I

transcripts in a murine model *in vivo*. In this same study, fusion to protamine of an antibody specific for the hormone receptor ERBB2 allowed siRNA targeting of cancer cells expressing that receptor[58].

A similar technology for specific targeted delivery is based on aptamer–RNAi chimaeras[59]. Aptamers are *in vitro*-evolved, synthetically prepared nucleic acids that selectively bind specific ligands. An RNA aptamer designed to bind prostate-specific membrane antigen (PSMA; also known as FOLH1) was linked to a *PLK1*-targeted siRNA, and binding of the aptamer to the PSMA receptor resulted in the selective delivery into prostate cancer cells of siRNAs that target pro-survival genes[59,60]. Intratumoral injection of the PSMA–*Plk1*-targeted siRNA or PSMA–*Bcl2*-targeted siRNA conjugates into a mouse xenograft model resulted in triggering of apoptosis, growth inhibition and tumour regression[59].

A different conjugation of an siRNA to vitamin-A-coupled liposomes succeeded in delivering antifibrotic siRNAs to hepatic stellate cells, which are produced in response to liver damage[61]. In this study, multiple siRNA treatments targeting collagen chaperone-encoding genes reversed liver fibrosis by preventing collagen deposition and increased survival in rats, providing a potential therapeutic approach to treating liver cirrhosis.

Also noteworthy is the recent report of libraries of lipid-like molecules (lipidoids) that can be selected for siRNA delivery to various tissues[62].

Viral delivery

An alternative means of triggering RNAi is through promoter-expressed siRNA sequences processed from shRNAs or miRNA mimics. The genes encoding these hairpin structures are most commonly inserted into the backbones of viral vectors under the control of Pol II or Pol III promoters. A potential advantage of vector delivery is that a single administration triggers long-term expression of the therapeutic RNAi. This is particularly appropriate for chronic viral diseases such as HIV and viral hepatitis.

Lentiviral vectors have been used successfully to deliver shRNA constructs in various mammalian systems. For example, it was shown that downregulation of an activated *Ras* oncogene by a lentiviral-delivered shRNA resulted in suppression of tumour growth in mice[63]. And downregulation of the expression of a mutant form of superoxide dismutase 1 (SOD1) in mouse models of amyotrophic lateral sclerosis delayed the onset of disease[64,65]. More recently, a lentiviral vector was used to deliver a *Smad3*-targeted shRNA for regeneration of satellite cells and repair of old tissue in aged and injured muscle[66]. Viral-vector expression of shRNAs has also been explored in mouse models of neurodegenerative disorders such as Huntington's disease and Alzheimer's disease[67].

To deliver genes to the central nervous system, adenoviral vectors have proved very useful. For instance, direct brain injection of an adenoviral vector expressing a shRNA directed against the mRNA encoding the polyQ-harbouring SCA1-encoding transcript of spinocerebellar ataxia type 1 was shown to be an effective treatment in a mouse model of this disorder[68].

Despite the successes of viral delivery, it is important to bear in mind that although some viruses are non-pathogenic, they are still potentially immunogenic. Another major concern with this technique is the risk of incurring mutations in viral sequences, causing insertional mutagenesis or triggering aberrant gene expression. However, viral vectors can transduce both dividing and non-dividing cells, yield a prolonged expression of the therapeutic gene and need not be delivered in large doses. Ultimately, any therapeutic gene when expressed in large quantities has the potential to cause toxicity and immunogenicity. Critical parameters such as tolerability, long-term expression, efficacy and the ability to regulate expression and targeting should be taken into consideration when choosing a delivery method. There is no ideal delivery system for every application; rather, the delivery method needs to be tailored to the application.

Clinical trials using RNAi to treat human diseases

For a new technology, siRNAs have moved into the clinic at an unprecedented pace. Some examples of the diseases and siRNA-targeting strategies that are currently under investigation are described below.

The first siRNA protocol granted investigational new drug (IND) status and tested in a human clinical trial is the vascular endothelial growth factor (*VEGF*)-targeted siRNA Bevasiranib (Acuity Pharmaceuticals, Philadelphia, Pennsylvania) for the treatment of wet age-related macular degeneration (see Table 2 for a summary of ongoing siRNA clinical trials). This involves the overgrowth of blood vessels behind the retina, and causes severe and irreversible loss of vision; it affects 1.6 million people in the United States alone, and it is predicted that 11 million individuals worldwide will have the disease by 2013. Preclinical studies of Bevasiranib in mice showed reduced neovascularization resulting from downregulation of *Vegf* expression after direct ocular injection of the siRNA[69]. This siRNA, which is now in a phase III trial, is also in a phase II clinical trial for the treatment of diabetic macular oedema. By the conclusion of these trials, several hundred patients will have received the siRNA treatments.

Two other companies are also focusing on siRNA-based treatments against macular degeneration: Merck's Sirna Therapeutics (San Francisco, California) with an siRNA (Sirna-027) that targets the VEGF receptor VEGFR1, and Quark Pharmaceuticals (Fremont, California) in collaboration with Silence Therapeutics (London and Berlin; previously SR Pharma), with one targeted against a hypoxia-inducible gene, *RTP801* (also known as *DDIT4*), that is known to be involved in disease progression. This siRNA, RTP801i-14, has been licensed to Pfizer, UK, which is now running a phase I/IIA clinical trial. Quark Pharmaceuticals has also received IND status for another preclinical trial, in which it is currently enrolling patients. This trial is for an siRNA targeting *TP53* mRNA (which encodes the protein p53), inhibition of which delays the induction of cell-death pathways and thereby reduces acute kidney injury after surgery.

Calando Pharmaceuticals (Pasadena, California), meanwhile, has initiated a phase I clinical trial for solid tumours using an siRNA that targets a subunit of ribonucleotide reductase (RRM2), an enzyme required for the synthesis of DNA building blocks. Importantly, this trial is the first to utilize receptor-mediated delivery of siRNAs, which are encapsulated in cyclodextrin particles decorated with transferrin. This results in uptake by cells expressing the transferrin receptor, which is highly expressed on cancer cell surfaces.

The clinical trials performed by Acuity Pharmaceuticals and Merck's Sirna Therapeutics successfully stabilized patients' conditions against further degeneration and improved their vision without adverse effects. These results engendered great optimism for intravitreal injection of siRNAs, but in a stunning turn of events a report by Kleinman *et al.* demonstrated that the observed decrease in vascularization could be a consequence not of an siRNA-specific effect on angiogenesis, but rather a nonspecific activation of Toll-like receptor 3 (TLR3) and subsequent activation of interferon-γ and interleukin 12, which, in turn, downregulate VEGF[70]. In other words, both the targeted and the control siRNAs mediated nonspecific inhibition of angiogenesis through a direct interaction of the siRNAs with TLR3. Cellular uptake is not necessary for this effect, and because TLR3 is involved in several other cellular pathways the finding has highlighted another level of concern for safe clinical use of siRNAs.

Alnylam Pharmaceuticals (Cambridge, Massachusetts) is a well-established siRNA-therapeutics company whose leading candidate siRNA, ALN-RSV01, is now in a phase II clinical trial. This siRNA targets respiratory syncytial virus — which affects almost 300,000 people every year in the United States alone — by silencing the virus's nucleocapsid 'N' gene, a gene essential to viral replication. ALN-RSV01 was the first antiviral siRNA to enter clinical trials, and trials will soon be expanded to paediatric patients. Thus far it has been shown to be effective and well tolerated. Recently, Alnylam Pharmaceuticals formed an exclusive alliance with Kyowa Hakko Kogyo to develop and commercialize ALN-RSV01 in Japan and other Asian countries.

Also in development at Alnylam Pharmaceuticals are siRNAs directed against genes implicated in hypercholesterolaemia, Huntington's disease (in a joint venture with Medtronic of Minneapolis, Minnesota), hepatitis C (in a joint venture with Isis Pharmaceuticals in Carlsbad, California), progressive multifocal leukoencephalopathy (in a joint venture with Biogen Idec of Cambridge, Massachusetts) and pandemic flu (in a joint venture with the Swiss company Novartis).

The International Pachyonychia Congenita Consortium (IPCC), in collaboration with TransDerm (Santa Cruz, California), has developed an siRNA to allow the correct production of keratin as a treatment for a rare skin disorder called pachyonychia congenita.

The City of Hope National Medical Center in Duarte, California, in collaboration with Benitec (Melbourne, Australia), has started a phase I trial for the treatment of AIDS lymphoma. This trial uses a Pol III promoter-expressed shRNA targeting the HIV *tat* and *rev* shared exons. The shRNA has been incorporated into an HIV-based lentiviral vector, which in turn has been used to insert the shRNA gene (along with two other RNA-based anti-HIV genes) into blood stem cells[71]. The gene-modified stem cells have been infused into HIV-positive patients in a trial that uses autologous bone marrow transplantation to treat AIDS-related lymphomas. Four patients have now been treated in this trial.

As indicated above, partnerships have become quite accepted in the field of siRNA biotechnology. These consortia are considerably increasing the capital available for these efforts and are shortening the time involved in commercializing siRNA-based drugs.

Some companies, such as Regulus Therapeutics (Carlsbad, California), have chosen to focus on miRNAs as therapeutic targets. Santaris Pharma in Hørsholm, Denmark, has recently started the first phase I trial to target a human miRNA (miR-122). In this trial, miR-122 is being targeted for downregulation with a locked nucleic acid (LNA) anti-miRNA (SPC3649). LNA is a backbone modification that enhances the hybridization of the oligonucleotide with its target and protects it from nuclease degradation. The approach is intended to treat hepatitis C virus infection because miR-122 facilitates replication of this virus in the liver[72,73]. Downregulation of miR-122 is also potentially useful in the treatment of hypercholesterolaemia. Targeting miRNAs expressed in the heart, such as miR-208, which regulates cardiac hypertrophy and fibrosis[74], may have an advantage, because in the medical field there is a considerable experience in delivering drugs directly into this organ.

Gain or loss of miRNA function has been linked to the onset and progression of various diseases[75–77]. Protein function can be regulated either directly or indirectly by miRNAs, and alterations in miRNA expression can have profound effects on gene regulation. In instances in which disease results from altered miRNA expression, it is conceivable that normal levels could be achieved, either by targeting the specific miRNA if expression is too high or by delivering a miRNA mimic if expression is too low. However, the specificity and efficacy of delivery systems would need to be improved for this goal to be accomplished. Moreover, correct modulation of the targeted miRNA's expression is not an easy task, and it is not clear whether one miRNA can be specifically targeted without affecting other miRNAs of the same family.

The regulatory complexities of miRNAs should also be taken into consideration when either ablation or restoration of miRNA function is being considered in a therapeutic setting. A single miRNA can regulate the levels of hundreds of proteins[78,79], raising cautionary flags about the consequences of downregulating or ectopically expressing even a single miRNA species.

The safety issue

The application of siRNAs to therapeutics has raised a number of concerns about their safety. After the initial excitement, a number of reports underscored potential drawbacks to this promising technology. The first warning came from a study that recorded the deaths of mice after Pol III promoter-driven expression of shRNAs in the liver[4]. The exact mechanisms leading to mortality are still under investigation, but seem to be due at least in part to saturation of the transport factor, exportin 5, that ferries miRNAs from the nucleus to the cytoplasm. There are now indications that other factors involved in the RNAi process can also be saturated by high-level expression of exogenous siRNAs, which can sequester them from their cognate cellular miRNAs. Because each cellular miRNA can potentially modulate the expression of several hundred genes[78,79], minor alterations in the miRNA pathway can have major consequences.

One strategy to mitigate this problem is to use the lowest possible concentration of siRNAs that provides therapeutic efficacy by designing the exogenous siRNAs to be Dicer substrates (by increasing their length). These RNAs enter the RNAi pathway upstream of the RISC at the step of Dicer cleavage, which facilitates passing the siRNA to AGO2 for selection of the guide strand, often resulting in enhanced RNAi at lower concentrations than can be achieved with the exogenous delivery of cognate 21-base siRNAs[80–83]. Although small amounts of siRNAs are not expected to saturate the RNAi machinery, they can compete with miRNAs for selective incorporation into the RISC[5]. The long-term consequences of such competition are poorly understood.

With the use of microarrays, it has become increasingly obvious that introducing foreign siRNAs into the cell alters the expression of non-target genes, as well as target genes[84,85]; as few as six or seven nucleotides complementary to the seed region could result in a specific off-target effect[86] through a miRNA-like mechanism. Because microarrays only reflect mRNA levels, they do not take into account any genes affected at the translational level, and so at present it is not clear how extensive the problem of off-target effects really is. Given that the application of synthetic siRNAs results in transient inhibition of gene expression, specific off-targeting may not be a major concern for many clinical applications. Nevertheless, appropriate toxicity testing should take into account the potential for a particular siRNA to target 3′ UTRs in non-target genes.

Some strategies can be used in siRNA design to minimize the problem of off-targeting. For instance, it has been shown that 2′-*O*-Me modifications[87] or DNA substitutions[88] in siRNA duplexes can significantly reduce off-target effects. It would also be valuable to improve antisense-strand selectivity by taking into account thermodynamic stability (see 'Superior designs for small molecules') or by blocking the 5′ phosphorylation of the sense strand[89].

RNAi is a widely conserved mechanism that may originally have evolved to combat viral infections. As such, it is perhaps not surprising that in some cases siRNAs can act as agonists of Toll-like receptors[90] and that specific sequence motifs, such as uridine-rich regions and guanosine- and uridine-rich regions, can induce cellular immune responses[6,7].

The ability of an siRNA to stimulate cellular immune responses is based not only on specific sequences but also on structure, the type of delivery system used and the cell type[7,91]. Although the immunostimulatory potential of siRNAs could be advantageous in certain circumstances[92], it is usually an unwanted outcome. The above-mentioned finding of the TLR3 response to non-sequence-specific modulation of VEGF or the VEGF receptor[70], as well as a separate report showing that a macrophage migration inhibitory factor (*Mif*)-targeted siRNA (in a murine model) and a nonspecific control siRNA increased the proliferation of breast cancer cells through activation of dsRNA-activated protein kinase (PKR)[93], raise serious concerns in interpreting the results of *in vivo* siRNA applications.

Although we have yet to reach a universal solution for avoiding all off-target effects, it is foreseeable that these problems will be overcome by the use of appropriate backbone modifications, as well as delivery systems that can mask RNAs from the receptors of the innate immune system[94].

Gazing ahead

Despite the technique's youth, the list of diseases for which RNAi is being tested as a therapeutic agent is extensive, and includes Parkinson's disease, Lou Gehrig's disease, HIV infection, wet age-related macular degeneration, type 2 diabetes, obesity, hypercholesterolaemia, rheumatoid arthritis, respiratory diseases and cancers. It is already a multimillion dollar business, projected to reach US$1 billion by 2010, and intellectual property rights will become an increasingly important concern in the coming years.

However, although much has been accomplished, obstacles remain that will hamper the race to the clinic. The ultimate goal of achieving RNAi-based therapies for life-threatening or debilitating diseases cannot be attained without improving the safety, effectiveness and reliability of RNAi-trigger delivery systems. The use of targeted delivery strategies that permit systemic delivery will be a big step towards fulfilling this difficult task. The development of new, noninvasive imaging methods to monitor the *in vivo* delivery of siRNAs, such as labelling with near-infrared dyes[95], will aid studies of tissue uptake and biodistribution.

Although RNAi is not yet an accepted therapeutic modality, the enormous interest in this phenomenon ensures that we will soon witness fast advances and new applications for RNAi-based therapies. Given the way that RNAi has transformed basic research and the unprecedented speed with which it has reached the clinic, the coming years promise to be exciting. ■

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